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P. Sassone-Corsi
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# Epigenetics, Brain and Behavior

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# Epigenetics, Brain and Behavior

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# Foreword

The ability to store information over long periods of time lies at the heart of cellular identity. This cellular 'memory' is encoded in the specific pattern of expressed genes and allows a cell to ensure that it "remembers" who it is and how it should move along elaborate pathways during cellular development and differentiation. Nerve cells become hard-wired during their differentiation, through changes that allow them to properly control cognitive and behavioral functions. How could one relatively fixed genetic blueprint permit flexibility to accommodate variability resulting from signals originated from environmental, dietary and other influences?

Neurons are submitted to an exceptional variety of stimuli and are able to convert these into high order functions, such as storing memories, controlling behavior, and governing consciousness. These unique properties are based on the highly flexible nature of neurons, a characteristic that is commonly thought to rely on the complex molecular machinery that controls gene expression. What lies at the heart of neuronal plasticity? Accumulating evidence points to epigenetics. This word originally indicated potentially heritable modifications in gene expression that do not involve changes in DNA sequence. Today this definition is much less strict, and epigenetic control is thought to include DNA methylation, histone modifications, histone variants, microRNA metabolic pathways and non-histone proteins modifications. Thus, while neuronal plasticity is rightly thought to be intimately associated to genomic control, it is critical to appreciate that there is much more to the genome than DNA sequence, permitting variability beyond the Watson-Crick double helix. The multitude of epigenetic mechanisms operating in all cells, but specifically in neurons, begs the question of how these may be coordinated.

Recent years have seen spectacular advances in the field of epigenetics. These have attracted the interest of researchers in many fields and evidence connecting epigenetic regulation to brain functions has been accumulating. Neurons daily convert a variety of external stimuli into rapid or long-lasting changes in gene expression. A variety of studies have centered on the molecular mechanisms implicated in epigenetic control and how these may operate in concert. It will be critical to unravel how specificity is achieved. Importantly, specific modifications

seem to mediate both developmental processes and adult brain functions, such as synaptic plasticity and memory. Many aspects of the research in neurosciences and endocrinology during the upcoming decade will be dominated by the deciphering of epigenetic control. The idea of the *IPSEN Colloque Medecine et Recherche* on 'Epigenetics, Brain and Behavior' held in Paris on April 18, 2011, was to indeed bring together some of the leaders in the field to discuss most recent developments and exciting new directions. The physiological implications of epigenetic regulation in the control of neuronal functions were highlighted, as well as the increasing understanding of the molecular mechanisms that operate within neurons to translate epigenetic control into long-lasting neuronal responses. These proceedings constitute a compendium of the most updated views in the field. We trust that it will be of use to those colleagues who will be picking up the challenge to unravel the epigenetic pathways that will ultimately allow future development of specific pharmacological strategies towards neurodegenerative pathologies.

Paolo Sassone-Corsi
Yves Christen

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# Nucleosome Remodelling and Epigenome Diversification

**Peter B. Becker**

**Abstract** Nucleosome remodelling factors utilize chemical energy to disrupt histone-DNA interactions in nucleosomes. They catalyze a variety of structural changes ranging from complete nucleosome disassembly and the exchange of histone variants to the sliding of intact histone octamers along DNA. The essential involvement of some remodelling complexes in neuronal transcription programs is due to lineage-specific subunits that assure the selective targeting to neuronal promoters. In addition, nucleosome remodelers may contribute to the faithful silencing of competing transcription programs by maintaining the integrity and regularity of the nucleosomal fiber as a prerequisite for higher order chromatin organization.

## 1 Introduction

Energy-dependent nucleosome remodelling provides a solution to a general problem that arises as a consequence of the chromatin organization of eukaryotic genomes. On the one hand, the packaging of DNA needs to be tight to protect the precious genetic information from damage and rearrangement. The concomitant compaction of DNA establishes a generally repressive environment, as DNA is largely rendered inaccessible to regulatory proteins. On the other hand, different parts of the genome need to be accessed in a highly regulated manner in response to metabolic, developmental and environmental signals. Access to DNA in chromatin is regulated by a dedicated class of nucleosome remodelling enzymes that catalyze

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reversible chromatin structure transitions by changing the histone-DNA interactions in individual nucleosomes – the fundamental unit of chromatin (Becker and Horz 2002; Clapier and Cairns 2009; de la Serna et al. 2006).

Nucleosome remodelers endow chromatin with structural flexibility and effectively render the nucleosomal organisation 'transparent' to the factors that need access to DNA, be it to regulate transcription or to repair damage. Because chromatin is a stable structure, remodelling reactions are energetically coupled to ATP hydrolysis. Nucleosome remodelers harbor a DNA-stimulated ATPase domain that permits translocation of the enzyme on DNA. Other domains on the same polypeptide and on associated subunits make contact with the histones or with linker DNA between adjacent nucleosomes. These contacts position the ATPase on the nucleosome such that the translocation disrupts critical histone-DNA contacts. Current concepts propose that conformational changes triggered by ATP binding, subsequent hydrolysis and product release displace a segment of DNA from the histone octamer's surface. The outcome of iterative reaction cycles – be it the removal of histones or the dislocation of intact histone octamers on DNA – largely depends on the properties of the remodelling enzyme itself (such as processivity, geometry, step size) and cooperating factors, such as histone chaperones. The mechanistic details of the remodelling reaction are not known but are the subject of intense study (for recent reviews, see Cairns 2007; Gangaraju and Bartholomew 2007; Racki et al. 2009).

About 30 ATPases exist in humans, and they are known or suspected to function as nucleosome remodelers through their sequence features (Hargreaves and Crabtree 2011). The ATPases themselves, the complexes they form and often also their functions have been conserved during evolution. The enzymes can be sorted into 24 different subfamilies according to sequence features (Flaus et al. 2006). Most of our current knowledge stems from just four families that are named after the lead enzyme that was first identified: SWI/SNF, ISWI, CHD and INO80 (Bao and Shen 2011; Kasten et al. 2011; Sims and Wade 2011; Yadon and Tsukiyama 2011). The ATPases combine with other subunits to form a large diversity of remodelling complexes. These subunits serve to regulate the activity of the machinery, to target the complex to specific sites of action and to integrate the remodelling reaction into physiological processes (Clapier and Cairns 2009). The complexity of these molecular machines has increased dramatically during metazoan evolution, and the full scope of the 'variations of a theme' that characterizes mammalian complexes, including subunit isoforms, variants and posttranslational modifications, can only be vaguely anticipated. With the discovery of mechanistic detail using model promoters, it is becoming increasingly clear that different remodelling principles may cooperate in a promoter-specific manner. For example, progesterone induction of the Mouse Mammary Tumor Virus promoter involves an initial recruitment of the ISWI-containing nucleosome remodeler NURF, which leads to displacement of the linker histone H1, which is followed by association of a BAF complex (see below) to catalyze the eviction of histones H2A/H2B (Vicent et al. 2011). A synergistic action of CHD7 and PBAF in orchestrating the transcription program of neural crest cells has not been explored in mechanistic detail (Bajpai et al. 2010).

The involvement of dedicated remodelers in lineage specification largely relies on their targeting to specific promoters, where they function as co-regulators (Chioda and Becker 2010; Ho and Crabtree 2010). I will use the example of the mammalian SWI/SNF-type BAF complexes to illustrate how the incorporation of cell type-specific subunits endows a generic remodelling machinery with selectivity for neuronal promoters. However, lineage-specific programs are defined not only by the activity of certain genes but also by the faithful silencing of competing transcription programs. In this context we increasingly appreciate the involvement of some remodelling enzymes in the diversification of the epigenome through assembly of repressive higher order structures. The ISWI-containing CHRAC/ACF complexes in *Drosophila* may serve to illustrate this principle.

## 2 Transcription Control: Harnessing Remodelers to Install Neuronal Differentiation Programs

Remodelling factors were initially identified in the search for principles able to 'open up' chromatin to allow regulatory factors to access their DNA binding sites. They frequently serve as cofactors that render promoters accessible and are, therefore, integral to most, if not all, transcription programs. Whereas the promoters of housekeeping genes are often characterized by intrinsic nucleosome depletion and are, therefore, less dependent on nucleosome remodelling, the highly regulated, 'conditional' promoters require remodelers to remove nucleosomes to facilitate interactions of the basal transcription machinery (Cairns 2009). In some instances, the linker histone H1 is the initial substrate for remodelling, as a prelude to further nucleosome rearrangements (Vicent et al 2011). Nucleosome removal may follow different pathways: either nucleosomes are directly disassembled in cooperation with histone chaperones (Clapier and Cairns 2009) or histone octamers may be moved along DNA to clear a particular sequence and even be used as a tool to lever out a neighboring histone octamer (Chaban et al. 2008; Engeholm et al. 2009). In any case, the extent of remodelling is local and often restricted to a single or perhaps very few nucleosomes. The remodelling reaction does not always lead to gene activation, since activators or repressors of transcription may equally profit from increased accessibility of DNA.

Remodelling enzymes are abundant constituents of nuclei in higher organisms. It has been estimated that an embryonic stem (ES) cell nucleus contains about 300,000 BRG1 molecules. Only a minority can be found associated with promoters or enhancers, suggesting that BAF complexes may regulate transcription at a distance or have additional functions (Ho et al. 2009). The collective abundance of remodelers suggests that they may - unlike metabolic enzymes – not turn over many substrate molecules and have non-enzymatic roles as well. Indeed, the fact that they are abundant chromatin constituents presenting many interacting surfaces suggests that their mere presence has a significant impact on chromatin organization (Korber and Becker 2011; Varga-Weisz and Becker 2006).

One of the best-studied remodelers is the SWI/SNF complex in yeast, an 11-subunit assembly that is targeted to many promoters through direct interactions with sequence-specific DNA binding factors (Workman 2006). SWI/SNF cooperates with other chromatin modifiers, notably acetyltransferases that acetylate the N-terminus of histone H3, thereby weakening electrostatic histone-DNA interactions (Eberharter et al. 2005; Narlikar et al. 2002). The remodelling ATPase SWI2/SNF2 carries a bromodomain that may interact with acetylated histones, thereby promoting the retention of the remodeller (Hassan et al. 2001; Kim et al. 2010).

The *Drosophila* homolog of SWI2/SNF2 is called BRM (Brahma) for historical reasons. It associates with 7–8 'Brahma-associated proteins' (BAP) to form complexes that loosely resemble the yeast SWI/SNF complex. Interestingly, two related complexes exist, BAP and PBAP, that share six subunits but differ in the signature subunits Osa, Polybromo and BAP170 (Mohrmann et al. 2004). The 'core complex' in the absence of these signature subunits is not functional. At the same time, they specify the preferential recruitment to distinct sets of target genes. The functions of the two complexes are in part redundant and partly distinct, but the basis for their gene selectivity has not been resolved yet (Moshkin et al. 2007).

The mammalian orthologs of Brahma are the highly related ATPases BRM and BRG1, and the proteins that associate with them to form a family of related remodelling machineries are termed 'BRG/BRM-associated factors' (BAFs; Hargreaves and Crabtree 2011). The BAF complexes resemble the original SWI/SNF complexes vaguely: they share five orthologous subunits, but contain at least another seven different subunits. A common theme of mammalian BAF complexes is their diversity, as different isoforms of several of subunits exist that associate with BAF complexes in mutually exclusive and combinatorial ways (Hargreaves and Crabtree 2011; Wu et al. 2009). This finding may explain why BAF complexes are involved in regulating very different gene expression programs, from the maintenance of the pluripotent state of ES cells to the highly differentiated profile of postmitotic neurons (Ho and Crabtree 2010). The involvement of a variety of chromatin remodelling principles particularly in neurogenesis has been summarized in a number of excellent recent review articles (Brown et al. 2007; Hargreaves and Crabtree 2011; Ho and Crabtree 2010; Yoo and Crabtree 2009), and we will limit the discussion here to variants of the BAF complexes that play roles in lineage specification and differentiation.

The differences between the BAF complex purified from ES cells (esBAF) and the complex isolated from postmitotic neurons (nBAF) are that the former never contains BRM or BAF170 but features BAF53a and BAF45d, and the latter may contain BRM in place of BRG1 and BAF170 instead of BAF155 and is characterized by the presence of the variants BAF53b and BAF45b/c (Lessard et al. 2007; Olave et al. 2002). The closely related BAF45 variants differ in their N-terminal Krüppel-like domain, which presumably does not directly function as a DNA binding domain. The most likely scenario is that the specificity subunits mediate interactions with different transcription factors that recruit the complexes to alternative sets of genes. The BAF53b subunit of nBAF contributes to tethering

the remodeler to promoters of genes that specify dendritic outgrowth, in addition to uncharacterized contacts of the complex with the dendritic regulators CREST (Wu et al. 2007). The tethering of BAF complexes to neuronal transcription factors may be modulated by antagonists, as illustrated by the case of *Xenopus* neuronal precursors, where the association of Geminin with BRG1 prevents association of the remodeler with neurogenin-type transcription factors (Seo et al. 2005).

Remarkably, the proliferating and self-renewing neural progenitor cells contain BAF complexes (npBAF) of intermediate subunit composition (containing BAF170, but not BAF45b or BAF53b; Lessard et al. 2007). Depending on the system, the BAF60c variant appears to also be characteristic of npBAF (Lamba et al. 2008). The switch between the different BAF complexes involves repression of BAF53a through microRNAs that are specifically activated in post-mitotic neurons and suppressed by the action of the transcription repressor REST in precursor cells (Yoo and Crabtree 2009). Continued expression of the progenitor signature subunits BAF45a and BAF53a prevents neuronal differentiation (Lessard et al. 2007; Yoo and Crabtree 2009). BAF53 variants are actin-related proteins (homologous to Arp4 in yeast) that bind ATP and are able to contact histones (Hargreaves and Crabtree 2011).

A further mechanism that contributes to BAF complex diversity is to generate neuron-specific BAF57 isoforms through differential splicing (Kazantseva et al. 2009).

The neurogenic role of specialized BAF complexes has been conserved during evolution, since an RNA interferences screen in flies identified BRM, BAP60 and BAP55 (the ortholog of BAP53) as important for correct dendrite routing of olfactory projection neurons (Seo et al. 2005).

## 3 Global Phenomena – Epigenome Diversification

The local action of nucleosome remodelers at promoters after recruitment by specific transcription factors illustrates most intuitively the role of remodelling complexes in executing cell type-specific gene expression programs. The recruiting ‘pioneer’ transcription factors in turn may profit from the global action of untargeted remodelers that are thought to transiently render nucleosomal DNA accessible through nucleosome mobilization (Becker 2002). In special cases, remodelers may cooperate with transcription factors to set a critical nucleosome position as a substrate for additional remodelling (Belikov et al. 2000).

However, the maintenance of cell-specific gene expression programs requires not only the activation of a critical set of genes but also the repression of alternative programmes. The stable repression of inappropriate genes is chromatin-based and relies on layers of chromatin organization ‘on top of’ the classical nucleosome fiber. The most prominent representatives of these ‘epigenetic’ repressors are members of the so-called Polycomb Group (PcG) system and a class of proteins that were first characterized as heterochromatin components (e.g., heterochromatin protein 1, HP1)

that participate in self-propagating and self-reinforcing molecular networks that render chromatin inactive. Nucleosome remodelling by the SWI/SNF complex is inhibited by either principle in vitro: the compaction of nucleosomal fibers by the Polycomb-repressive Complex (PRC1) may render the substrate inaccessible (Francis et al. 2001). HP1 proteins compete with the SWI/SNF complex for a critical epitope on nucleosomes (Lavigne et al. 2009).

The differentiation of pluripotent cells in a developing organism and the corresponding selective realization of a particular gene expression program is accompanied by a corresponding diversification of the epigenome – the differential packaging of the genome into permissive and repressive domains. Pluripotent cells are characterized by a particularly open chromatin conformation, which reflects the plasticity of the state (Melcer and Meshorer 2010). It comes as no surprise that nucleosome remodelers also help to maintain this state. For example, the depletion of the single-subunit remodeler CHD1 in mouse ES cells leads to the inappropriate accumulation of heterochromatin and a decreased capacity for self-renewal (Gaspar-Maia et al. 2009). It is unclear to what extent the underlying mechanism has been conserved during evolution. In *Drosophila,* the ATPase is important for chromatin formation in the paternal nucleus of the zygote. In CHD1 mutants, the paternal pronucleus fails to specifically incorporate the histone variant H3.3 and the resulting deranged chromatin prevents proper fusion with the maternal pronucleus and subsequent nuclear cleavage (Konev et al. 2007).

In this context, the case of the *Drosophila* CHRAC/ACF complexes may be instructive (Chioda and Becker 2010; Chioda et al. 2010). These remodelers are closely related, as they both contain the ATPase ISWI and the 'signature subunit' ACF1. CHRAC is distinguished from ACF by the additional presence of two small histone-fold proteins. The in vitro activities of CHRAC and ACF are very similar: their ATP-dependent nucleosome remodelling leads to the sliding of intact histone octamers on DNA (Längst and Becker 2001). Catalyzed nucleosome sliding endows chromatin with 'transparency' towards DNA binding proteins, at least in vitro, as random nucleosome movements lead to transient exposure of any sequence. However, one of the remarkable features of these remodelers is that they not only are able to render DNA in a nucleosomal fibre accessible but, conversely, can also even out discontinuities in nucleosome density. They convert irregular successions of nucleosomes into evenly spaced nucleosomal arrays (Becker 2002). Regular nucleosomal arrays – but not arrays with gaps - can fold into 30 nm-type fibers, which may promote the assembly of epigenetically repressed structures of the Polycomb- and HP1-types mentioned above. This possibility is supported by the observation of 'sloppy' embryonic chromatin, reduced heterochromatin formation and leaky chromatin-based repression by the Polycomb system in the absence of ACF1 (Fyodorov et al. 2004).

Hints of a dependence of heterochromatin silencing on the integrity of the underlying nucleosomal array can already be found in yeast. Curiously, the fission yeast *S. pombe* does not contain a remodelling ATPase of the ISWI type, but the CHD3/4-related ATPase Mit1 could fulfill similar functions. Mit1 is required for the phasing of nucleosomes next to *active* promoters (Lantermann et al. 2010).

Remarkably, however, deletion of the *mit1* gene also alters the regularity of nucleosome arrangements at the silent mating type loci and alleviates heterochromatin silencing, leading to the hypothesis that repressive heterochromatin is linked to intact nucleosome arrays (Sugiyama et al. 2007).

The expression of ACF1 – and therefore the presence of the remodelling factors it defines – is developmentally regulated (Chioda et al. 2010). ACF1 it very abundant in preblastoderm embryos, which are characterized by developmental and epigenetic plasticity, and is strongly down-regulated in post-mitotic, differentiated cells. ACF1 can still be detected in committed precursor cells, notably the primordial germ stem cells and the larval neuroblasts. ACF1 is, therefore, most abundant when the epigenome is not yet fully defined. Depletion of ACF1-containing remodelers appears to impair the diversification of the epigenome. The recurrent idea is that repressive chromatin structures must be grounded on an intact nucleosomal fiber (Korber and Becker 2011). Ectopic expression of ACF1 in the neuronal precursors of the larval *anlagen* (the eye/antenna imaginal disc) perturbs the programmed differentiation into the photoreceptors of the compound fly eye (Chioda et al. 2010). One may speculate that inappropriate expression of ACF1 might cause a 'regression' of epigenome diversification to the unstructured, uncommitted state.

## 4 Conclusion

Nucleosome remodelers are involved in all aspects of nuclear metabolism. They contribute to cellular differentiation processes through at least two very different principles. On the one hand, they may function as transcription co-regulators that are recruited to locally 'remodel' chromatin at regulatory sites to implement cell type-specific transcription programs. On the other, they may function as global organizers of the chromosomal fiber as a prerequisite for the subsequent assembly of repressive chromatin that prevents the interference of competing programs.

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# Synaptic Epigenesis and the Evolution of Higher Brain Functions

**Jean-Pierre Changeux**

**Abstract** The epigenesis theory of development can be traced back to William Harvey (1651), who stated, in contrast to contemporary preformation views, that the embryo arises by "the addition of parts budding out from one another." The word epigenesis was subsequently used by Conrad Waddington (Nature 150:563–565, 1942) to specify how genes might interact with their surroundings to produce a phenotype. This is also the meaning we adopted in our paper, Theory of the Epigenesis of Neuronal Networks by Selective Stabilization of Synapses (Changeux et al. Proc Nat Acad Sci U S A 70:2974–2978, 1973), according to which the environment affects the organization of connections in an evolving neuronal network through the stabilization or degeneration (pruning) of labile synapses associated with the state of activity of the network. This definition contrasts with the recent and more restricted sense of the status of DNA methylation and histone modification in a particular genomic region. The synapse selection theory was introduced to deal with two major features regarding the genetic evolution of the human brain : 1) the non-linear increase in the organizational complexity of the brain despite a nearly constant number of genes ; and 2) the long postnatal period of brain maturation (ca. 15 years in humans), during which critical and reciprocal interactions take place between the brain and its physical, social and cultural environment. This theory will be evaluated and updated in the framework of the recent human/primate genome data, analysis of gene expression patterns during postnatal development, brain imaging of cultural pathways, such as those for language learning, and current views about the neural bases of higher brain function, in particular the global neuronal workspace architectures for access to consciousness (see Dehaene and Changeux Neuron 70:200–227, 2011).

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## 1 Introduction

The term ‘epigenesis’ (or ‘epigenetics’) should not be seen as applying only to molecular mechanisms of chromatin modification and the regulation of gene expression. Well before the term was hijacked by molecular biologists, we had applied it to a higher level of adaptation: the selection and stabilization of synaptic connections in the central nervous system by activity, through which the animal learns to adapt to its environment (Changeux et al. 1973; Changeux and Danchin 1976). The dramatic differences in structure and function between the brains of humans and lower mammals largely result from the high proportion of the connections in the human brain that are established during post-natal life. The continuation of the epigenetic mechanisms of synaptic selection throughout life are of particular significance in humans as they both underpin and are driven by social and cultural learning. Here I review the evidence for this type of epigenesis in the developing and adult nervous system.

One essential aspect of postnatal development in the human brain is the establishment of long-range connections between different cortical areas, especially those linking the parieto-temporal-cingulate cortices with the prefrontal lobes, which are the centers for decision making, rational thinking and social interaction. According to our global neuronal workspace hypothesis (Dehaene et al. 1998), these connections are a prerequisite for accessing consciousness. We are further testing the importance of their epigenetic stabilization in two animal model systems: genetically modified mice in which genes for a nicotinic receptor subunit have been deleted and quantitative evaluation of the expression profiles and activity dependence of 12,000 genes in the rat from embryo to adult. Some of these genes are involved in the regulation of chromatin modifications, providing a bridge between the synaptic and molecular levels of epigenetic regulation.

## 2 Defining Synaptic Epigenesis

The term epigenesis was first applied to the nervous system in the context of the selection and stabilization of synapses during development (Changeux et al. 1973; Changeux and Danchin 1976). The growth of axons towards their targets – the dendrites of target neurons in the central nervous system or muscle cells in the periphery – involves cell-surface recognition molecules, possibly ones unique to the specific category of connections. The axon terminals branch exuberantly at first but then are pruned back in response to neuronal activity, both intrinsic spontaneous neuron firing and that evoked by external inputs. Depending on the state of activity of the target neuron, some synapses are eliminated while others are strengthened and stabilized (Fig. 1). In post-natal life, an important part of the activity in the network results from inputs from the environment and so the epigenetic selection of synapses represents learning in the network as the organism is shaped to fit its environment. In this sense, learning can be considered as a Darwinian process, because it depends on elimination of the ‘unfit’ synapses.

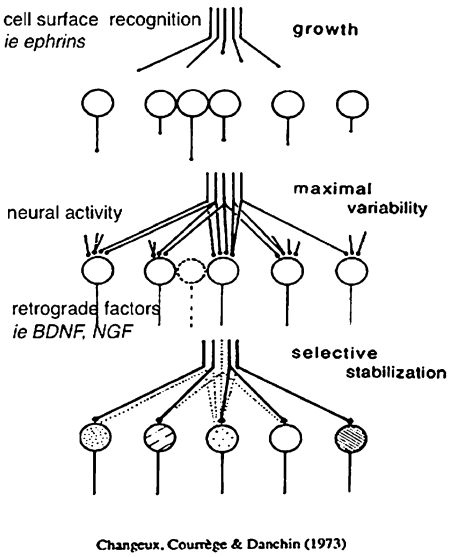


**Fig. 1** Schematic representation of the hypothesis of epigenesis by selective stabilization of synapses. A nesting of many of such elementary steps occurs in the course of development. (from Changeux 1985)

The word epigenetic was first used by William Harvey (1651), who, noting how complexity of form gradually emerges during embryogenesis, applied it to the description of how an embryo arises by "the addition of parts budding out from one another." In more modern times, the developmental biologist Conrad Waddington (1942) used the word to specify how genes interact with their surroundings to produce a phenotype. He conceived the concept of the 'epigenetic landscape' to illustrate how external events, some random, combine with inherited information coded in the genes to produce members of a species that, although recognizably related, have individual characteristics. His usage bridges the gap between synaptic epigenesis as defined above and the more molecularly oriented definition used in the introduction to this volume, i.e., mechanisms that permit variability beyond the Watson-Crick double helix.

One factor of considerable importance in discussing epigenetic mechanisms is that the brain is unique among organs in the specificity and number of its multiple connections between neurons (around $10^{15}$ in humans), a level of organization not found among, for example, the cells of the liver. Establishing and maintaining this huge connectivity requires a set of cellular processes and molecular mechanisms employed only in the nervous system.

## 3 Social and Cultural Evolution

Synaptic epigenesis is of particular significance in the context of the human brain because it enables social and cultural evolution (Changeux 1985). Looking at the multiple levels of variability and their timescales within the human brain, we see,

first, the variability of the genome, which underlies the biological evolution of the ancestors of *Homo sapiens* over millions of years. Next, on the ontogenetic or developmental level, is the epigenetic variability of neuronal networks and the connections that are established over days to years, depending on the species; beyond this, in humans, are the dynamics of thought, based on the variability of spontaneous/evoked neural activity and on the efficacy of synaptic connections, which operate in the 1–100 msec domain.

Finally, social and cultural evolution is associated with variable synaptic efficacy and the establishment of extracerebral memories in the form of spoken, written and pictorial material, with a time range of 100 msec to thousands of years. Spoken language and, perhaps even more significantly, writing are seminal innovations that distinguish humans from other primates; they drove the development of modern civilization and have probably also been central to the expansion of human mental capacities. Writing can be traced back to abstract cave drawings, dated around 30,000 BCE; clay counting tokens are known from Mesopotamia (9,000 BCE) and the first pictograms from Ur are from around 4,000 BCE. More important for my thesis, language and writing rely on epigenetic cultural transmission framed within a robust genetic envelope. The huge postnatal increase in the size of the human brain – the adult brain weights five times that of the newborn infant and about 50 % of the adult brain's connections develop after birth (Huttenlocher and Dabholkar 1997; Bourgeois 1997) - offers the developing brain the opportunity for intense social and cultural interactions.

Paradoxically, the evolution of the genome has lagged well behind the increase in brain complexity during mammalian evolution (Venter et al. 2001). The mouse, the rat and the monkey have approximately the same number of genes as a human (20,000-25,000). Yet the mouse has only $40 \times 10^6$ neurons, whereas the human brain contains in the range of $50–100 \times 10^9$. Gene sequences have not increased in complexity in parallel with the increase in complexity of the brain. Yet what happened? The explanation for this disparity, in my view, lies in a few genetic events that favored the prolonged and extensive post-natal increase in neuronal branching and synaptic connectivity in humans and its modulation by extended epigenetic responses to the environment.

## 4 Synaptic Selection and Stabilization

The extension of the post-natal period of development in humans has been essential for the genesis and internalization of culture, as well as for the acquisition and transmission of individual experience. In rats, the maximum synaptic density is reached within a few weeks after birth, whereas in humans it takes over three years. Moreover, rats show little loss of synapses after maximum density is reached, whereas in humans there is a steady decline until the total number stabilizes about the time of puberty (Huttenlocher and Dabholkar 1997; Bourgeois 1997; Petanjek et al. 2011), reflecting the initial exuberance and later pruning of

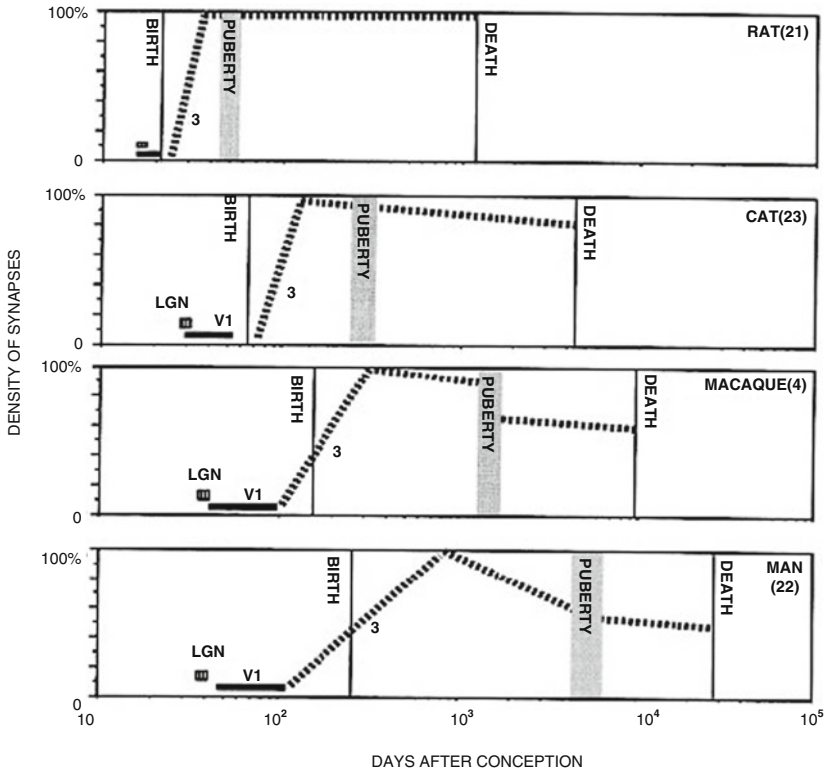


**Fig. 2** Postnatal evolution of the total number of cortical synapses in different mammalian species. Note the significant decrease in the global envelope of synapses taking place before puberty in humans. (from Changeux 2004)

connections (cat and monkey show intermediate stages of this process as life span and infant dependency increase; Fig. 2). This major distinction between lower mammals and humans has to be borne in mind when using rats and mice as models for human psychiatric and neurological conditions. When *H. sapiens* appeared in Africa about 100,000 years ago, half the average life span of around 30 years would have been taken up with building the brain. And, of course, in contemporary humans, the process of synaptic refinement goes far beyond puberty: learning is lifelong (Petanjek et al. 2011).

The decline in synaptic numbers during childhood reflects learning by selection. The initially exuberant connections are refined by activity in the network as some synapses are reinforced and others are eliminated (Fig. 1). Some early demonstrations of this process include work on motor neurons innervating muscle cells (Benoit and Changeux 1975, 1978; O'Brien et al. 1977; Henderson et al. 1986; Gouzé et al. 1983; Bourgeois et al. 1986), the establishment of retinal inputs to the cat lateral geniculate body (Stretavan et al. 1988); and studies in many other systems (Luo and O'Leary 2005; Wu et al. 2012; Kano and Hashimoto 2011; Ko et al. 2011).

These and many other studies have shown that, when neuronal activity is artificially modified, synaptic pruning is altered. In particular, at variance with the classical Lamarckist-constructivist scheme (Quartz and Sejnowski 1997), blocking the activity maintains a high number of connections: it is activity that enhances synaptic elimination (Benoit and Changeux 1975, 1978; Stretavan et al. 1988; Luo and O'Leary 2005). As I have said in the past « to learn is to eliminate » (Changeux 1985).

More recent work has revealed detailed mechanisms involved in synaptic plasticity, including processes of molecular selection by changes in the diffusion dynamics of receptors in the synaptic membrane while receptors maintain a stable density under the nerve ending (Triller and Choquet 2008), and allosteric state transitions in the NMDA receptor trappping the diffusible D1 dopamine receptor that result in an increase in spines bearing dopamine receptors (Scott et al. 2006), which is a plasticity phenomenon possibly altered in neuropsychiatric disorders.

Disruption of synapse selection and connectivity can also be seen in several human neurodevelopmental disorders. For instance, various mutations linked to autism are in genes that are involved in synapse formation/stabilization, such as NEUROLIGINS 3/4, NEUREXIN 1 and SHANK 3, which code for synaptic adhesion and stability proteins (Bourgeron 2009, this volume). The dynamics of synapse stabilization is altered in Fragile X mental retardation by expanded CGG repeats in the FMR1 gene, which produces a protein that interferes with the Rac1 pathway and controls actin cytoskeleton dynamics (Mandel and Biancalana 2004; Castets et al. 2005). Changes in synaptic pruning have been associated with the onset of schizophrenia, a disease that has been linked to the susceptibility genes ERBB4, SLC1A3, RAPGEF4 and CIT28; the last is also involved in bipolar disorder (Karlsgodt et al. 2008). Links with NEUREXIN 1, which is, as mentioned, involved in synapse formation/stabilization, have also been reported (Cook and Scherer 2008).

The theory of the epigenesis of neuronal networks by selective stabilization of synapses (Changeux et al. 1973) was introduced to account for the interactions that take place between the brain and its physical, social and cultural environment in the course of development; this theory therefore accounts for the variability in the brain's connectivity and in behavior between individuals, associated with the variability of the environment. Such an epigenetic variability of brain anatomy would be superimposed on that created by the variability of the genome. Another critical feature of the theory is that, conversely and unexpectedly, it may account for the constancy of some behaviors despite epigenetic variability. The same learning input may not stabilize the same connective patterns in different individuals but nevertheless result in the same behavior.

This idea was originally stated as the 'variability theorem' (Changeux et al. 1973) that « different learning inputs may produce different connective organizations and neuronal functioning abilities, but the same behavioral abilities. » This finding is particularly evident in the brains of genetically identical individuals that, contrary to expectation, do not show identical nerve organization. To give a simple example, the exact branching patterns of identified motor neurons in a parthenogenetic fish, *Poecilia formosa*, have been established by electron microscopy. This pattern was found to vary significantly not only between genetically identical individuals

(identical twins) but also on the left and right sides of the same animal. Yet the fishes all swim the same way (Levinthal et al. 1976). Thus, to some extent, the development of the fine details of the connectivity pattern includes a stochastic element - chance plays a part in determining exactly which synapses survive - yet the behavior may nevertheless remain constant between individuals. At a much higher level of complexity, humans with language areas located either in the left or right hemispheres, or in both, are indistinguishable by the way they speak or think! There is no contradiction between epigenetic « Darwinian » selection and the occurrence of behavioral universals.

One of the critical inputs that may contribute to synapse selection is reward (Thorndike 1911; Hull 1943; Skinner 1981). Positive reward is signalled by neurons in the brain stem that release dopamine in the frontal cortex, whereas serotonin neurons signal negative reward, or punishment (Dehaene and Changeux 1991, 2000). The evolution of connectivity through selection has been tested using a network simulation that can learn to do specific tasks when given simple positive and negative rewards (Gisiger et al. 2005). Before learning, the connectivity in the network is diffuse and homogeneous and task completion is unsuccessful; after learning, the selected connections form a coherent and organized network that can complete tasks successfully. Further work should establish the actual contribution of reward to synapse selection in the course of development.

## 5 Epigenetics and Higher Brain Function

As already discussed, post-natal selection is essential for establishing the neuronal circuits serving culturally acquired behaviors such as writing and reading. By painstakingly reconstructing the lesions in the brains of patients with various deficits, Dejerine (1901) was able to identify a variety of defects associated with learning written language. One such lesion caused an inability to read but left writing intact, a condition called pure alexia; the reverse lesion produced agraphia without alexia. These observations can be understood as evidence for separate epigenetic “cultural » circuits” for reading and writing that are laid down in childhood.

More recently, the circuits involved in literacy have been examined using brain imaging. They confirm Dejerine’s pionneering insight. These studies (Castro-Caldas et al. 1998) took advantage of behavioral evidence of different phonological processing in illiterate vs literate subjects. During repetition of real words, the literate and illiterate groups performed similarly and activated similar areas of the brain. In contrast, illiterate subjects had more difficulty correctly repeating pseudowords and did not activate the same neural structures as literates. Comparison of PET scans from illiterate and literate groups showed a considerable shift in activation. For instance, in a pseudowords–words contrast, activation in the literate group was stronger in the right frontal opercular–anterior insular region, left anterior cingulate, left lentiform nucleus and anterior thalamus/hypothalamus compared with the illiterate group (Castro-Caldas et al. 1998; Carreiras et al. 2009).

The connectivity used for reading and writing can thus be seen as an epigenetic appropriation of existing circuitry. Scans from ex-illiterates showed substantial similarity to those from literates, showing that the ability to acquire these cultural imprints persisted into adulthood (Dehaene et al. 2010).

Among the cortical connections established in post-natal life are the long-range tracts between the frontal areas and other brain cortical areas (including sensory ones) that determine and organize action (see Pugliese et al. 2009). Some years ago, we proposed that these long-range connections, by broadcasting signals to multiple brain areas, yield subjective experience (Dehaene et al. 1998; Dehaene and Changeux 2011). We termed this the 'global neuronal workspace' hypothesis: by allowing sensory inputs – seeing, hearing and so on – global access to many brain areas, the long-range connections provide a structural basis for the global experience known as conscious access.

Long-range connections mostly originate from the pyramidal neurons in cortical layers II and III and are specially abundant in the prefrontal cortex (von Economo and Koskinas 1925). Particularly important are the connections with the prefrontal areas, the part of the cortex involved in planning, decision making, thought and socialization, that have evolved most dramatically between mice and humans (for a more detailed review of the global neuronal workspace hypothesis and its applications, see Changeux 2006; Changeux and Lou 2011; Dehaene and Changeux 2011).

One question we are currently asking is whether these long-range connections are especially vulnerable to some pathologies. As mentioned above, the onset of schizophrenia has been linked to susceptibility genes coding for several proteins involved in synaptic pruning (Karlsgodt et al. 2008; Cook and Scherer 2008). Significantly, the long-range connections might be affected differentially by susceptibility mutations that are known to affect synaptogenesis in general (Scott-Van Zeeland et al. 2010). This vulnerability might result, for instance, from a very low nucleo-cytoplasmic ratio and/or changes in the long-distance transport of essential cellular components along the axons, which could explain the specificity of the schizophrenic phenotype as distinct from the mental retardation expected from a global deficit in synaptogenesis.

One way we are testing this vulnerability is by comparing the dendritic branching of pyramidal neurons in wild-type mice with animals lacking the β2-subunit of the nicotinic acetylcholine receptor (Ballesteros-Yáñez et al. 2010). Loss of this subunit prevents the high-affinity binding of the neurotransmitter acetylcholine and, as a consequence, mice lacking the β2-subunit show a characteristic behavioral deficit: their exploratory drive, which is one of the most cognitive aspect of mouse behavior, is reduced, although their navigation abilities, a more automatic activity, are unaffected. Even though establishing a fair analogy between mouse behavior and human psychology may look far-fetched, it has been hypothesized that the mice might possibly be showing an alteration in elementary conscious access (see Avale et al. 2011; reviewed in Changeux 2006).

So far, we have not been able to examine the long-range projections but we have measured the complexity of the basal dendrites of 650 neurons from layer III in seven areas from the cortex of wild-type and β2−/− mice. In agreement with

Elston's (2003) work on the monkey, the neurons in the wild-type mice show a gradient of complexity: those in anterior areas have longer dendrites, larger dendritic fields and more spines. Such a complexity gradient may be interpreted in terms of the global neuronal workspace hypothesis as resulting from denser long-range connectivity originating from prefrontal areas. In contrast, the neurons in the knock-out mice are fairly uniform throughout the cortex, implying that the neurons in the frontal areas are not receiving as many inputs from the sensory areas as they do in the wild-type animals. In other words, the lack of nicotinic receptor activation seems to epigenetically enhance a selective loss of long-range connectivity.

These studies on mice may be relevant to a possible effect of chronic nicotine use on long-range connectivity. In humans, diffusion tensor imaging, which allows the meaurement of the location, orientation, and anisotropy of the white-matter tracts in the brain, has shown reduced integrity in the frontal white matter in people who are cocaine dependent or who abuse heroin. The same method has revealed that prenatal and adolescent exposure to tobacco smoke alters the development of the microstructure of the white matter, with increased fractional anisotropy in right and left frontal regions and in the genu of the corpus callosum (reviewed in Changeux and Lou 2011). These observations suggest that nicotine may also act directly on white matter and there is electrophysiological evidence that supports a direct action of nicotine on axon conduction, possibly at the level of the node of Ranvier (reviewed in Changeux 2010). Thus there is support for direct control of the global neuronal workspace by nicotine at the white matter level. This work further implies that drugs of abuse like nicotine may interfere with the functioning of the long-range cortical connections, so addicts may lose some conscious control of their actions.

## 6 Where Next?

We are turning back to the conventional tools of molecular genetics to map the expression patterns of genes involved in synaptic epigenesis throughout the life span of the rat. Using chip technology, the changes in expression of 12,000 genes in the cortex have been analyzed by computational methods at stages from embryonic day 16 through birth, puberty and adulthood to old age at post-natal day 90 (Tsigelny et al. submitted). One dramatic change is at birth, when many genes active in the embryo are switched off and thousands of other genes become active, just as the animal goes through the most radical change in its environment that it is ever likely to encounter.

One fascinating aspect of this study is that some of the activated genes are involved in the regulation of chromatin modifications, which links the level of synaptic epigenesis that I have been discussing here back to the molecular mechanisms that are being detailed by other contributors to this volume.

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# Plasticity of the Circadian System: Linking Metabolism to Epigenetic Control

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**Abstract** Control of a large variety of neuronal, behavioral and physiological responses has been linked to the circadian clock, made possible through a transcriptional regulatory network that governs a significant portion of the genome. The harmonic oscillation of gene expression is paralleled by critical events of chromatin remodeling that appear to provide specificity and plasticity in circadian regulation. Accumulating evidence shows that the circadian epigenome appears to share intimate links with cellular metabolic processes, suggesting that the circadian epigenome might constitute the functional basis of tissue-specificity within biological pacemakers, specifically in neurons.

## 1 Circadian Clock and System Biology

A wide variety of physiological functions, including sleep-wake cycles, body temperature, hormone secretion, locomotor activity and feeding behavior, depend on the circadian clock, which is a highly conserved system that enables organisms to adapt to common daily changes, such as the day-night cycle and food availability (Schibler and Sassone-Corsi 2002). Based on evidence accumulated over several decades, it is safe to state that circadian rhythms possibly represent the most appropriate example of systems biology (Cermakian and Sassone-Corsi 2000).

In mammals, the anatomical structure in the brain that governs circadian rhythms is a small area consisting of ~15,000 neurons localized in the anterior hypothalamus, called the suprachiasmatic nucleus (SCN; Ralph et al. 1990; Welsh et al. 1995). This 'central pacemaker' receives signals from the environment and coordinates the oscillating activity of peripheral clocks, which are located in almost

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all tissues (Morse and Sassone-Corsi 2002; Schibler and Sassone-Corsi 2002; Yamazaki et al. 2000; Yoo et al. 2004). One important feature of the circadian clocks is that they are self-sustaining; circadian oscillations that are intrinsic to each cell can occur autonomously, without any environmental signal. However, because the period of the oscillations is not exactly 24 hours, the endogenous clock needs to be synchronized by external cues, a process called 'entrainment.' External cues (also known as zeitgebers) reset the system daily and thereby prevent the endogenous clock from free-running out of phase. The predominant external cue of the central clock is light (Quintero et al. 2003). In mammals, specialized cells in the retina detect the light signal, which is then transmitted to the SCN via the retinohypothalamic tract (RHT; Bellet and Sassone-Corsi 2010; Freedman et al. 1999; Gooley et al. 2001). At the level of SCN neurons, the light signal stimulates a cascade of signalling pathways that lead to the activation of a transcriptional program that involves immediate early genes and clock-controlled genes (CCGs). These gene expression events are associated with specific histone modifications leading to chromatin remodelling (Crosio et al. 2000). Peripheral tissues also contain functional circadian oscillators that are self-sustaining at the single cell level, but they do not respond to light–dark cycles and they appear to require other physiological stimuli to sustain their circadian rhythms.

Importantly, a lesion of the SCN in rodents disrupts the circadian periodicity in peripheral tissues, whereas SCN transplantation into SCN-ablated arrhythmic animals restores this dysfunction (Lehman et al. 1987; Ralph et al. 1990). Additional experiments in which the transplantation approach was applied to peripheral tissues demonstrated a hierarchical dominance of the SCN over clocks in peripheral tissues (Pando et al. 2002). To date, however, the means by which the SCN communicates with peripheral tissues to sustain and synchronize their cycles is not clear. Several observations support the idea that communication may be exerted by a combination of neuronal signals through the autonomic nervous system and humoral factors, of which glucocorticoids and retinoic acid are the most likely candidates (Antle and Silver 2005; Green et al. 2008). Furthermore, peripheral rhythms in mammals are affected by other SCN-independent stimuli (Yoo et al. 2004). Although light is the main stimulus that entrains the central pacemaker, peripheral clocks can themselves be entrained by food (Stokkan et al. 2001), probably through modifications of hormonal secretion or metabolite availability. Restricted access to food can reset the phase of peripheral oscillators, with little if any effects on the SCN central pacemaker (Damiola et al. 2000).

Another important environmental cue is temperature (Roenneberg and Merrow 2005). Temperature compensation is one of the most prominent features of the circadian system, as it allows the integration of moderate variations in ambient temperature that do not affect the period length of circadian oscillation. Nevertheless, low-amplitude temperature cycles can synchronize the circadian clocks in peripheral tissues in mammals, independently of the central clock (Brown et al. 2002). Altogether, these notions underscore the intimate links between the circadian clock and cellular metabolism (Green et al. 2008; Eckel-Mahan and Sassone-Corsi 2009).

## 2 The circadian transcriptome

At the heart of the molecular network that constitutes the circadian clock are the core transcription factors CLOCK and BMAL1, which heterodimerize and direct transcriptional activation of CCGs by binding to E-box sites on their promoters (Fig. 1). Among these CCGs, CLOCK and BMAL1 also direct transcription of their own repressors, period (PER) and cryptochrome (CRY) family members, creating a tightly self-regulated system (Sahar and Sassone-Corsi 2009). During the day, transcription of PER and CRY is high, leading to protein translation of the circadian repressors and resulting in formation of the inhibitory complex with CLOCK and BMAL1 that abolishes transcription of CCGs. The degradation of PER and CRY alleviates transcriptional repression and allows CLOCK:BMAL1-mediated transcription to again proceed, establishing an oscillatory rhythm in circadian gene expression. Additional levels of circadian regulation exist with the orphan nuclear receptors RORα and REV-ERBα, which activate and repress transcription of the *Bmal1* gene, respectively (Shearman et al. 1997; Albrecht et al. 1997), and the possibility that clock proteins can be regulated in a post-translational manner, as in the case of SUMOylation of BMAL1 (Cardone et al. 2005).

While the basic molecular organization and conceptual design of these autoregulatory loops are common to both SCN and peripheral tissues, it is intuitive that the circadian functions and outputs of SCN, liver or skeletal muscle are vastly divergent, begging the question as to how the pacemakers intrinsic to these tissues may differ. Indeed, the property of circadian synchronicity in culture is unique to SCN neurons; although each has a sustained circadian cycle, cultured cells from peripheral tissues do not display concerted oscillations (Stephan and Zucker 1972). On the other hand, it is reasonable to speculate that tissue-specific transcriptional regulators may contribute or intersect with the clock machinery. Several genome-wide array analyses have focused on determining the proportion and specificity of cycling transcripts (Masri and Sassone-Corsi 2010). The first remarkable finding indicated that ~10 % of all expressed genes in any tissue are under circadian regulation (Tousson and Meissl 2004; Schibler and Sassone-Corsi 2002; Moore and Eichler 1972; Stephan and Zucker 1972). This unexpectedly high proportion of circadian transcripts suggests that the clock machinery may direct widespread events of cyclic chromatin remodeling and consequent transcriptional activation/repression. Furthermore, genome-wide studies comparing the central SCN pacemaker and peripheral tissues, such as the liver, revealed that between 5 and 10 % of cycling genes where identical in both tissue types (Akhtar et al. 2002; Panda et al. 2002). A recent analysis covering 14 mouse tissues identified ~10,000 known genes showing circadian oscillations in at least one tissue. The number of common genes showing circadian oscillation in multiple tissues decreased drastically as the number of tissues included in the comparative analysis increased, with only 41 genes displaying circadian oscillation in at least 8 of 14 tissues (Yan et al. 2008). These findings underscore the presence of molecular interplays between the core clockwork, which can be assumed to be common to all tissues, and cell-specific transcriptional systems.

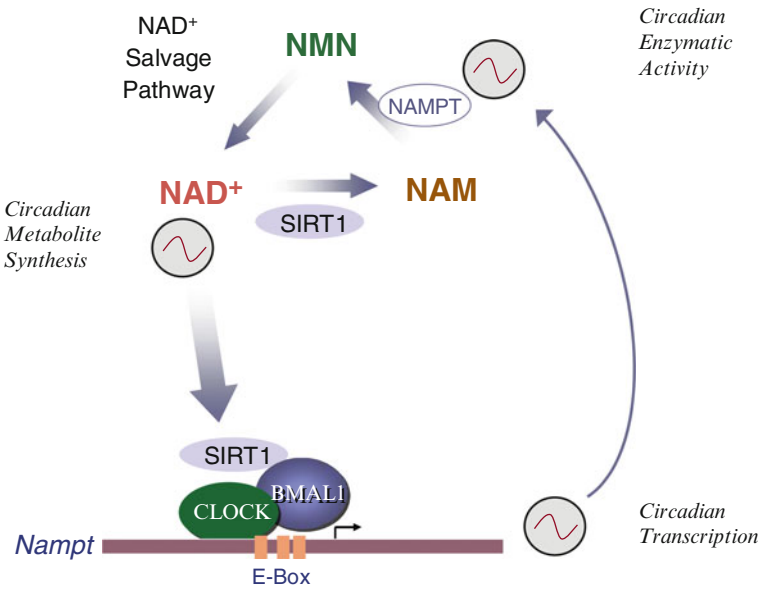


**Fig. 1** Linking cellular metabolism to the circadian clock; the metabolite $NAD^+$ oscillates in a circadian manner. A series of studies (Nakahata et al. 2009; Ramsey et al. 2009) demonstrated that the circadian clock machinery controls the cyclic synthesis of $NAD^+$ through control of the $NAD^+$ salvage pathway. The gene encoding the enzyme NAMPT, the rate-limiting step in the $NAD^+$ salvage pathway, contains E-boxes and is controlled by CLOCK-BMAL1. A crucial step in the $NAD^+$ salvage pathway is controlled by SIRT1, which also contributes to the regulation of the *Nampt* promoter by associating with CLOCK-BMAL1 in the CLOCK chromatin complex (Masri and Sassone-Corsi 2010). Thus, the feedback transcriptional loop of circadian regulation is intimately linked to an enzymatic feedback loop. Through this regulation, SIRT1 controls the cellular levels of its own coenzyme, $NAD^+$. [$NAD^+$, nicotinamide adenine dinucleotide; NAM, nicotinamide; NMN, nicotinamide mononucleotide; ~, oscillation of CCGs (*Nampt*) and metabolites ($NAD^+$)]

Taking into consideration the recent view of the mammalian circadian clock as a transcriptional network (Baggs et al. 2009; Ueda et al. 2005) through which the oscillator acquires plasticity and robustness, it is reasonable to speculate that the clock network contributes to physiological responses by intersecting with cell-specific transcriptional pathways. This notion has been demonstrated in the way that the circadian machinery interplays with other signaling-responsive transcription factors, such as CREB (Travnickova-Bendova et al. 2002).

## 3 Chromatin Remodeling and Epigenetic Control of Circadian Expression

How does the complex organization of chromatin cope with the cyclic regulation of circadian genes? Several histone modifications contribute to chromatin remodeling and, thereby, to the control of a large array of nuclear processes (Cheung et al. 2000;

Borrelli et al. 2008). The N-terminal domains of histones are subjected to several types of covalent modifications, such as acetylation, phosphorylation, methylation and others. A number of these modifications have been associated with distinct chromatin-based outputs. For example, position-specific modifications of the histone H3 N-terminal tail have been coupled with transcriptional regulation (Lys9/Lys14 acetylation, Ser10 phosphorylation), transcriptional silencing (Lys9 methylation), histone deposition (Lys9 acetylation) and chromosome condensation/segregation (Ser10/Ser28 phosphorylation). It is believed that specific signaling pathways lead to distinct histone modifications (Cheung et al. 2000), suggesting that various physiological stimuli translate into differential chromatin remodeling (Borrelli et al. 2008).

Histone acetylation has been shown to play a pivotal role in the modulation of chromatin structure associated with transcriptional activation (Berger 2007). In support of this notion, a wide variety of nuclear proteins involved in transcriptional control possess intrinsic histone acetyltransferase (HAT) activity. We have found that one of these is the master regulator CLOCK, whose HAT function is essential for circadian control (Doi et al. 2006). Accumulating evidence from various laboratories indicates that CLOCK is an enzyme that directs circadian changes in chromatin remodeling.

We have shown that chromatin remodeling is coupled with circadian clock function (Crosio et al. 2000) and that the protein CLOCK functions as an enzyme that induces chromatin remodeling (Doi et al. 2006). This previously unforeseen activity of a core clock factor has several, far-reaching biological implications. CLOCK is a HAT that preferentially modifies histone H3 in position Lys14, a site where addition of an acetyl group results in stimulation of gene expression. Thereby, CLOCK acts as an enzyme that globally modifies genome functions by inducing the opening of chromatin structure and allowing transcriptional activation. Our ongoing experiments demonstrate that CLOCK can be regulated by intracellular signaling, thereby connecting chromatin remodeling to circadian physiological responses. In addition, the enzymatic activity of CLOCK is not restricted to histones (Hirayama et al. 2007); our findings indicate that CLOCK acetylates its own partner, BMAL1. This modification occurs at one unique lysine residue in position Lys537 of the protein and it is essential for circadian rhythmicity (Hirayama et al. 2007; Nakahata et al. 2008). Recent data indicate that the histone methyltransferase MLL1 directs the cyclic tri-methylation of the H3K4 site on circadian promoters and directs the recruitment of the CLOCK:BMAL1 complex (Katada and Sassone-Corsi 2010).

The recent discovery that the activity of SIRT1, a longevity-associated protein belonging to a family of nicotinamide adenine dinucleotide ($NAD^+$)–activated histone deacetylases (Haigis and Guarente 2006), oscillates in a circadian fashion broadens our knowledge about the communication between the circadian clock and metabolism but also reveals a void in our understanding about the molecular support for such interplay. SIRT1 counterbalances the HAT function of CLOCK by deacetylating both K14-H3 and BMAL1 (Nakahata et al. 2008). SIRT1 demonstrates an oscillation in activity, impinging back on the circadian clock by

altering BMAL1 acetylation and CLOCK:BMAL1-induced gene transcription (Nakahata et al. 2008). The discovery of metabolite oscillations during the yeast metabolic cycle (Murray et al. 2007), combined with evidence of circadian sirtuin activity, allows speculation as to whether metabolites such as $NAD^+$ themselves serve a preponderant role in the cellular link between metabolism and the circadian clock (Fig. 1).

## 4 $NAD^+$ as a Central Circadian Regulator

Circadian oscillation of SIRT1 activity suggests that cellular $NAD^+$ levels may oscillate. Using accurate mass spectrometry/liquid chromatography measurements, our laboratory has confirmed this notion by demonstrating that $NAD^+$ levels indeed oscillate in serum-entrained mouse embryon fibroblasts (MEFs) and liver (Nakahata et al. 2009; Ramsey et al. 2009). Circadian clock controls the expression of nicotinamide phosphoribosyltransferase (NAMPT), a key rate-limiting enzyme in the salvage pathway of $NAD^+$ biosynthesis. CLOCK, BMAL1 and SIRT1 are recruited to the NAMPT promoter in a time-dependent manner. The oscillatory expression of NAMPT is abolished in *clock*/*clock* mice, which results in drastically reduced levels of $NAD^+$ in MEFs derived from these mice (Nakahata et al. 2009). These results make a compelling case for the existence of an interlocking of the classical transcriptional feedback loop that controls the circadian clock with an enzymatic loop, wherein SIRT1 regulates the levels of its own cofactor (Fig. 1).

The oscillation of $NAD^+$ levels begs the question of whether the activity of other $NAD^+$-dependent enzymes may be regulated in a circadian manner. In this respect, one class of enzymes appears to occupy a privileged position: the poly(ADP-ribose) polymerases (PARPs), which have been shown to functionally interact with SIRT1 (Kolthur-Seetharam et al. 2006). PARP-1, the most well-characterized PARP, is activated by DNA damage and plays a role in DNA repair. Since increased activity of PARP depletes the intracellular pool of $NAD^+$, this may lead to reduced SIRT1 activity and cell death (Kolthur-Seetharam et al. 2006). Given the direct control of SIRT1 deacetylase activity by $NAD^+$, circadian regulation of $NAD^+$ levels appears to be a critical regulatory mechanism controlling circadian rhythms, metabolism and cell growth. Interestingly, altered NAMPT levels have been implicated in metabolic disorders and cancer, and FK866, a highly specific NAMPT inhibitor that abolishes $NAD^+$ circadian oscillations and thereby SIRT1 cyclic activity, is used to control cell death in human cancer tissues. These results suggest that a direct molecular coupling exists between circadian clock, energy metabolism and cell survival. Future studies will reveal the precise function of circadian control of SIRT1 activity in the regulation of metabolism.

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# Epigenetics and the Environmental Regulation of Genomic Structure and Function: Implications for Health

**Carine I. Parent, Tie-Yuan Zhang, and Michael J. Meaney**

**Abstract** There are numerous examples in the behavioral sciences of the enduring effects of early experience on neural and immune function. In this article we review the emerging evidence for epigenetics as a candidate mechanism for these effects. Epigenetics refers to functionally relevant modifications to the genome that do not involve a change in nucleotide sequence. Such modifications include chemical marks that regulate the transcription of the genome. There is now evidence that environmental events can directly modify the epigenetic state of the genome. Thus studies with rodent models suggest that, during both early development and in adult life, environmental signals activate intracellular pathways that directly remodel the "epigenome," leading to changes in gene expression that modulate neural and immune function. These studies define a biological basis for the interplay between environmental signals and the genome in the regulation of individual differences in behavior, cognition, and physiology.

Psychology and psychiatry have seen a major transition in personality theory. Personality traits were thought to emerge solely under the influence of "nurture." The postnatal family environment was considered as the primary force in the development of personality. This perspective changed dramatically with the integration of the biological sciences into personality psychology. First, evolutionary approaches established that the brain, like any other organ, and its development are subject to selective forces. Second, behavioral genetics (Ebstein 2006;

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Kendler 2001; Plomin and Rutter 1998) provided evidence for a relationship between genomic variation and both personality and mental health. Although efforts to quantify the independent contribution of genetic and environmental influences are fraught with complications (e.g., gene–environment interactions, nongenomic mechanisms of inheritance), measures of concordance in specific traits between monozygotic and dizygotic twins, among other approaches, suggest an influence of genetic variation. Indeed, it is impossible to imagine that the function of brain cells occurs independently of sequence-based variations across the genome in regions that code for the RNAs and proteins that regulate neuronal functions.

Genomic variation at the level of nucleotide sequence is associated with individual differences in personality and thus with vulnerability and resistance to a wide range of chronic illnesses (Ebstein 2006; Meyer-Lindenberg and Weinberger 2006; Rutter 2007). The challenge for psychology and neuroscience is one of conceptually integrating findings from genetics into the study of personality to further our understanding of the basis for individual differences in vulnerability to mental illness and thus of pathophysiology. The challenge for psychoneuroimmunology also involves the integration of genetics into the study of immune function to further our understanding of the development of vulnerability and resistance to immune-related diseases. How and under what conditions does genomic variation influence the brain and immune development and function? How might relevant findings from the field of genetics influence the design of public policy and therapies in psychology and medicine?

Genes encode for RNAs and proteins, not function, which is a product of the interaction between environmentally driven cellular signals and the genome. The effects of genetic variation are thus contextually determined and best considered as probabilistic. The objective of this review is to describe recent advances in molecular biology, notably in the field of epigenetics, and to suggest that epigenetic mechanisms are ideal candidate mechanisms for the effects of environmental signals, including events such as social interactions, on the structure and function of the genome (Harper 2005; Meaney 2010). We suggest that epigenetics can account, in part, for instances in which environmental events occurring at any time over the life span exert a sustained effect on genomic function and phenotype.

Epigenetic signals refer to a series of chemical modifications to the DNA or to regions surrounding the DNA. The transcriptional activity of the genome is regulated by signals, which are transcription factors that physically bind to specific DNA sites. The importance of epigenetic mechanisms lies in their ability to regulate the ease with which transcription factors can access the DNA. Epigenetic signals can thus determine the capacity for environmental regulation of the genome. There is emerging evidence for the idea that epigenetic marks are directly altered in early life by environmental events and thus influence the development of individual differences in specific neural functions that underlie cognition and emotion. More recent studies suggest that dynamic alterations in these same epigenetic signals are crucial for the synaptic remodeling that mediates learning and memory. Further environmental events also lead to individual differences in immune function and in the propensity to develop immune-related disorders. Similar epigenetic

mechanisms are responsible for the differentiation of immune cells into different subtypes that are involved in the eradication of different pathogens. Thus, epigenetics provides a remarkable insight into the biology that governs the function of the genome in response to environmental signals.

## 1 Regulation of Glucocorticoid Receptor Expression

The neurotransmitter serotonin (5-hydroxytryptamine; 5-HT) regulates glucocorticoid receptor gene transcription in hippocampal neurons (Mitchell et al. 1990, 1992; Weaver et al. 2007). This effect is dependent upon the binding of the transcription factor nerve growth factor–inducible factor A (NGFI-A) to a specific binding site on the exon $1_7$ glucocorticoid (GR) promoter. The importance of this interaction can be precisely defined. For example, mutating a single nucleotide (i.e., a cytosine to adenine conversion) in the region of the promoter that normally binds NGFI-A abolishes the ability of NGFI-A to associate with the exon $1_7$ promoter and eliminates the effect of NGFI-A on gene transcription (Weaver et al. 2007). However, the ability of NGFI-A to bind to the exon $1_7$ promoter is regulated by another protein, a transcriptional cofactor, the CREB-binding protein, that is activated by the same 5-HT-cyclic adenosine monophosphate (cAMP)/cyclic nucleotide–dependent kinases (PKA)-signaling cascade that results in the increased levels of NGFI-A. The CREB-binding protein is a histone acetyltransferase. The association of the CREB-binding protein with the exon $1_7$ promoter is accompanied by an increase in the acetylation of a specific lysine on the tail of histone 3 of the exon $1_7$ promoter (Weaver et al. 2004, 2007). Thus, 5-HT activates both NGFI-A and the CREB-binding protein. Interestingly, NGFI-A and the CREB–binding protein physically associate with one another prior to DNA binding. The CREB–binding protein acetylates histones associated with the exon $1_7$ promoter, enhancing the ability of NGFI-A to bind and activate gene transcription.

Environmental signals alter 5-HT activity. Indeed, the effect of 5-HT on glucocorticoid receptor expression reflects the dependency of gene transcription on signals that derive from environmental events (note that the relevant environmental event may be internal or external to the organism; e.g., a change in the availability of glucose, an electrical impulse, or a social interaction). Such effects underlie the dynamic interdependence of gene and environment. However, developmentalists are also familiar with more enduring environmental influences - instances where experience in early life has shaped neural development and function in a manner that is sustained into adulthood. Such effects are considered to be the basis for environmental influences over the development of individual differences. In certain cases, the sustained effects of early experience associate with structural alterations in neural circuits that mediate specific functions. However, more recent studies suggest a form of environmentally regulated plasticity that exists at the level of genome and its associated chromatin. Such plasticity involves a variety of epigenetic marks that associate with DNA and/or histone proteins. These studies suggest

that environmental events alter the activity of specific intracellular signals that modify the nature of the epigenetic marks at specific sites in the genome, leading to sustained effects on gene expression and thus neural function.

Studies of development are replete with examples of the environmental programming of gene expression, such that a variation in the early environment associates with changes in gene expression and biological function that persist into adulthood. In the rat, for example, prenatal nutrient deprivation or enhanced exposure to hormonal signals associated with stress stably alter, or program, the activity of genes in the liver and other sites that are associated with glucose and fat metabolism, including the gene for the glucocorticoid receptor (Bateson et al. 2004; Gluckman and Hanson 2004, 2007; Jirtle and Skinner 2007; Meaney et al. 2007; Seckl and Holmes 2007). These findings are assumed to represent instances in which the operation of a genomic region in adulthood varies as a function of early environmental influences. The results of recent studies suggest that such "programming" effects can derive from gene–environment interactions in early life that lead to a structural alteration of the DNA, which in turn mediates the effects on gene expression as well as more complex levels of phenotype (Jirtle and Skinner 2007; Meaney 2007; Meaney and Szyf 2005). These studies were performed in rodents but were inspired by the vast literature reporting the pervasive effects of family environment on health outcomes in humans (Repetti et al. 2002). No less compelling are the results of studies on "maternal effects" in plants, insects, reptiles, and birds showing that variations in nongenomic signals of maternal origin associate with sustained effects on the phenotype of the offspring (Cameron et al. 2005; Mousseau and Fox 1998; Rossiter 1998).

The objective of these studies is to examine the biological mechanisms whereby variations in mother–infant interactions might directly influence gene expression and behavior (Meaney 2001). Such studies focus on variations in maternal behavior that lie within the normal range for the Norway rat and that occur in the absence of any experimental manipulations (i.e., naturally occurring variations in mother–pup interactions). Variations on maternal care in the rat are studied with simple observations of animals in their home cages (Champagne 2008; Champagne et al. 2003). One behavior, pup licking/grooming (LG), emerges as highly variable across mothers. Pup LG is a major source of tactile stimulation for the neonatal rat that regulates endocrine and cardiovascular function in the pup (Hofer 2005; Levine 1994; Schanberg et al. 1984). The question then was whether such variations in pup LG might directly alter the development of individual differences in behavior and physiology. For the studies reviewed here, the focus was on the development of individual differences in defensive responses.

Subsequent findings revealed considerable evidence for the effect of maternal care on the behavioral and endocrine responses to stress in the offspring. The male or female adult offspring of mothers that naturally exhibited increased levels of pup LG (i.e., the offspring of high-LG mothers) showed more modest behavioral and endocrine responses to stress compared to animals reared by low-LG mothers (Caldji et al. 1998; Francis et al. 1999; Liu et al. 1997; Menard et al. 2004; Toki et al. 2007; Weaver et al. 2004). Specifically, the offspring of high-LG mothers

showed reduced fearfulness and more modest hypothalamic-pituitary-adrenal (HPA) responses to stress. Cross-fostering studies, where pups born to high-LG mothers were fostered at birth to low-LG mothers (and vice versa), revealed a direct relationship between maternal care and the postnatal development of individual differences in behavioral and HPA responses to stress (Caldji et al. 2000, 2003; Francis et al. 1999; Weaver et al. 2004). In these studies, the rearing mother determined the phenotype of the offspring. Thus variations within a normal range of parental care can dramatically alter phenotypic development in the rat. Finally, experimental interventions that increased or decreased pup LG produced the expected developmental effect on responses to stress in the offspring (Francis et al. 1999; Toki et al. 2007).

The effects of maternal care on the development of defensive responses to stress in the rat involve alterations in the function of the corticotrophin-releasing factor (CRF) systems in selected brain regions. The CRF system furnishes the critical signal for the activation of behavioral, emotional, autonomic, and endocrine responses to stressors (Bale and Vale 2004; Koob et al. 1994; Plotsky et al. 1989). As adults, the offspring of high-LG mothers showed decreased CRF expression in the hypothalamus as well as reduced plasma ACTH and glucocorticoid responses to acute stress by comparison to the adult offspring of low-LG mothers (Francis et al. 1999; Liu et al. 1997; Weaver et al. 2004, 2005). Circulating glucocorticoids act at glucocorticoid receptor sites in corticolimbic structures, such as the hippocampus, to regulate HPA activity. Such feedback effects commonly inhibit hypothalamic CRF synthesis and release. The high-LG offspring showed significantly increased hippocampal glucocorticoid receptor expression, enhanced glucocorticoid negative feedback sensitivity, and decreased hypothalamic CRF levels (Liu et al. 1997). Importantly, pharmacological manipulations that blocked the effect of the glucocorticoid receptor eliminated the maternal effect on the HPA response to stress, suggesting that the differences in hippocampal glucocorticoid receptor expression are directly related to those at the level of HPA function.

Pup LG is a major source of tactile stimulation for the neonate. Experimental models that directly applied tactile stimulation, through the stroking of the pup with a brush, provided direct evidence of the importance of tactile stimulation derived from pup LG. Thus, stroking pups over the first week of life increased hippocampal glucocorticoid receptor expression (Jutapakdeegul et al. 2003) and dampened behavioral and HPA responses to stress (Burton et al. 2007; Gonzalez et al. 2001). Likewise, manipulations of lactating mothers that directly increased the frequency of pup LG also increased hippocampal glucocorticoid receptor expression and decreased HPA responses to stress (Francis et al. 1999, Toki et al. 2007). Manipulations, notably stressors imposed on the mother, that decreased pup LG were associated with increased behavioral and HPA responses to stress and were associated with decreased hippocampal glucocorticoid receptor expression and increased hypothalamic expression of CRF (Champagne and Meaney 2006; Fenoglio et al. 2005).

The offspring of the high-LG and low-LG mothers also differed in behavioral responses to novelty (Caldji et al. 1998; Francis et al. 1999; Zhang et al. 2004).

As adults, the offspring of the high-LG mothers showed decreased startle responses, increased open-field exploration, and shorter latencies to eat food provided in a novel environment. There were also behavioral differences in response to more precise forms of threat. Thus, the offspring of low-LG mothers showed greater burying of an electrified probe in the defensive burying paradigm (Menard et al. 2004). These differences in behavioral responses to stress are associated with altered activity in the CRF system that links the amygdala (and bed nucleus of the stria terminalis) to the noradrenergic cells of the locus coeruleus (Caldji et al. 1998; Zhang et al. 2004).

These studies suggest that variations in maternal care "program" stable changes in gene expression, producing individual differences in behavioral and neuroendocrine responses to stress in adulthood. The findings provide a potential mechanism for the influence of parental care on vulnerability/resistance to stress-induced illness over the life span. But the critical issue is simply that of how maternal care might stably affect gene expression. How are variations in the interactions between the mother and her offspring biologically embedded so as to stably alter the activity of specific regions of the genome?

The molecular processes that lead to the initiation of gene transcription involve modifications to the histone proteins that form the core of the nucleosome through the addition of acetyl groups to the N-terminal tail of the histone proteins. Such modifications open chromatin, permitting transcription factor binding and the activation of gene transcription. A second level of regulation occurs not on the histone proteins but rather directly on the DNA. Indeed, the classic epigenetic alteration is that of DNA methylation, which involves the addition of a methyl group (CH3) onto cytosines in the DNA (Bird 1986; Holliday 1989; Razin and Riggs 1980). DNA methylation in regulatory regions of the genome is associated with the silencing of gene transcription. The presence of the methyl group attracts a class of proteins known as methylated-DNA binding proteins (Klose and Bird 2007) that attract repressor complexes, including active mediators of gene silencing such as HDACs. HDACs prevent histone acetylation and favor a closed chromatin state that constrains transcription factor binding and gene expression. Compounds that inhibit HDACs can thus increase transcription from methylated DNA.

The methylation of DNA in mammals is an active biochemical modification that selectively targets cytosines and is achieved through the actions of enzymes, DNA methyltransferases, that transfer the methyl groups from methyl donors. Until recently, it was thought that DNA methylation patterns on the genome were primarily overlaid upon the genome only during early periods in embryonic development. Indeed, DNA methylation is considered to be a fundamental feature of cell differentiation, which involves silencing certain regions of the genome in a cell-specific manner. Such processes define the function of the cell type within the CNS (e.g., Fan et al. 2005). Such events occur early in development and are considered to be highly stable, such that dedifferentiation (whereby a cell loses its specialization) is rare and often is associated with organ dysfunction. This perspective was reinforced by findings showing that an alteration of DNA methylation at critical genomic targets (i.e., tumor suppressors) was associated with cancer (Eden et al. 2003; Feinberg 2007;

Laird 2005). Thus DNA methylation was considered both unique to early periods in development and mostly irreversible except under pathological conditions. Studies in neuroscience and immunology served to revise this perspective. Interestingly, in each discipline, the differentiation and function of cell subtypes showed considerable plasticity over the life span as a function of exposure to novel stimuli: environmental signals in the case of neurons and antigens in the case of immune cells (Renaudineau et al., 2010).

The issue for neurobiology is not only the processes by which cells specialize as neurons but also why neurons in one individual function differently from those of another, or how the functional properties of neurons might vary over the life span as a function of experience (i.e., activity-dependent neuronal plasticity). There is now considerable evidence in neuroscience and other fields, including immunology and endocrinology/metabolism, that the state of DNA methylation at specific genomic sites is indeed dynamic, even in adulthood (Bird 2007; Jirtle and Skinner 2007; Meaney and Szyf 2005). Moreover, alterations in DNA methylation are emerging as a candidate mechanism for the effects of early experience in individual differences in neural function, immune function and in learning and memory. Thus, recent studies reveal that DNA methylation patterns are actively modified in fully differentiated cells, including, and perhaps especially, neurons, and that such modifications can occur in animals in response to cellular signals driven by environmental events (Jirtle and Skinner 2007; Meaney and Szyf 2005; Sweatt 2009). For example, variations in the diet of mice during gestation or later in development, such as the early postweaning period, can stably alter the methylation status of the DNA (Cooney et al. 2002; Waterland and Jirtle 2003; Waterland et al. 2006; Whitelaw and Whitelaw 2006). Likewise, both mature lymphocytes (Bruniquel and Schwartz 2003; Murayama et al. 2006) and neurons (e.g., Champagne 2008; Champagne et al. 2006; Lubin et al. 2008; Martinowich et al. 2003; Sweatt 2009) show changes in the DNA methylation patterns at critical genomic regions in response to environmental stimuli that stably alter cellular function.

## 2 Epigenetics and the Social Environment

The critical feature of the maternal effects described above is that of persistence. The differences in the frequency of pup LG between high- and low-LG mothers are limited to the first week of postnatal life. The tactile stimulation associated with pup LG increases 5-HT activity in the hippocampus. In vitro studies with cultured hippocampal neurons showed that 5-HT acts on 5-$HT_7$ receptors to initiate a series of intracellular signals that culminate with an increase in the expression of NGFI-A as well as in the CREB-binding protein. Comparable effects occur in vivo. Manipulations that increase pup LG by lactating rats resulted in an increased level of cAMP as well as NGFI-A (Meaney et al. 2000). Pups reared by high-LG mothers showed increased NGFI-A expression in hippocampal neurons as well as an increased binding of NGFI-A to the exon $1_7$ promoter sequence

(Weaver et al. 2007; Zhang et al. 2006; Hellstrom et al. 2012). Moreover, the binding of NGFI-A to the exon $1_7$ promoter sequence is actively regulated by mother–pup interactions, such that there is increased NGFI-A bound to the exon $1_7$ promoter immediately following a nursing bout, but not at a period that follows 25 minutes without mother–pup contact (Zhang et al. 2006; Hellstrom et al. 2012).

NGFI-A and the CREB-binding protein form a complex that binds directly to the exon $1_7$ promoter sequence and actively redesigns the methylation pattern at this region of the genome (Weaver et al. 2004, 2007). Thus, as adults, the offspring reared by high-LG mothers showed very modest levels of methylation at the 5′ CpG of the NGFI-A consensus sequence.

Neither the 5′ nor the 3′ CpG site within the NGFI-A binding region is methylated in hippocampal neurons from fetal rats, whereas both sites are heavily methylated on the day following birth, with no difference as a function of maternal care (Weaver et al. 2004). These findings reflect de novo methylation. However, between the day following birth and the end of the first week of life, the 5′ CpG is 'demethylated' in pups reared by high-LG, but not low-LG, mothers. This difference persists into adulthood. Importantly, the period over which the demethylation occurs falls precisely within that time when high- and low-LG mothers differ in the frequency of pup LG; the difference in pup LG between high- and low-LG mothers is not apparent in the second week of postnatal life (Caldji et al. 1998; Champagne 2008; Champagne et al. 2003).

The demethylation of the 5′ CpG site occurs as a function of the same 5-HT-activated signals that regulate glucocorticoid receptor gene expression in cultured hippocampal neurons (Weaver et al. 2007). Thus, in cultured hippocampal neurons treated with 5-HT, which mimics the extracellular signal associated with maternal LG, the 5′ CpG site is demethylated; there is no effect at the 3′ CpG site. The binding of NGFI-A to the exon $1_7$ site is critical. Hippocampal neurons treated with an antisense or siRNA to NGFI-A showed neither a demethylation of the 5′ CpG site nor an increase in glucocorticoid receptor expression (Weaver et al. 2007). Likewise, site-directed mutation of the NGFI-A site (C to A at the 3′ CpG site) completely abolished the binding of NGFI-A to the exon $1_7$ promoter and prevented the demethylation of the 5′ CpG. Finally, the infection of hippocampal neurons with a virus containing a nucleotide construct engineered to express high levels of NGFI-A produced increased levels of the exon $1_7$ transcript and of mature glucocorticoid receptor mRNA, the demethylation of the 5′ CpG of the exon $1_7$ promoter sequence and increased glucocorticoid receptor expression (Hellstrom et al., 2012).

These studies raise an interesting issue in understanding the re-programming of DNA methylation in post-mitotic cells. If DNA methylation blocks transcription factor binding and the 5′ CpG site of the exon $1_7$ promoter is heavily methylated in neonates, then how might maternally activated NGFI-A bind to and remodel the exon $1_7$ region? Levels of the transcription factor–specific protein-1 (SP-1) and the CREB-binding protein are also increased in the hippocampus of pups reared by high-LG mothers (Weaver et al. 2007; Zhang et al., 2010; Hellstrom et al. 2012). The exon $1_7$ promoter contains a DNA sequence that binds SP-1 and overlaps with that for NGFI-A. SP-1 can apparently actively target both methylation and

demethylation of CpG sites (Brandeis et al. 1994). The 5′CpG site is the region of overlap in the binding sites. The CREB-binding protein acts as a histone acetyltransferase, potentially permitting the binding of transcription factors such as NGFI-A and SP-1. Increasing histone acetylation can lead to transcription factor binding at previously methylated sites and the subsequent demethylation of these regions (Fan et al. 2005; Cervoni and Szyf 2001; Szyf et al. 2005). Thus, we suggest that the binding of this complex of proteins - NGFI-A, the CREB-binding protein, and SP-1 - is critical in activating the process of demethylation. The results to date are certainly consistent with this model, although we have yet to establish the identity of the process responsible for the demethylation of the 5′ CpG site.

These findings suggest that maternally induced increases in hippocampal NGFI-A levels initiate the remodeling of DNA methylation at the regions of the DNA that regulate glucocorticoid receptor expression. The NGFI-A transcription factor binds to multiple sites across the genome. If NGFI-A-related complexes target demethylation, then one might assume that other NGFI-A-sensitive regions should show a maternal effect on DNA methylation and gene expression comparable to that observed with the glucocorticoid receptor. Zhang and colleagues (2010) showed that the hippocampal expression of the GAD1 gene that encodes for glutamic acid decarboxylase, an enzyme in the production of the neurotransmitter GABA, was increased in the adult offspring of high-LG mothers. This effect was associated with altered DNA methylation of an NGFI-A response element in a manner comparable to that for the glucocorticoid receptor gene. Moreover, as with the effect on the glucocorticoid receptor, an in vitro increase in NGFI-A expression mimicked the effects of increased pup LG. The function of GABAergic neurons in the limbic system was also regulated by maternal care (Caldji et al. 1998, 2000, 2003) and was a major target for anxiolytic agents. These findings are likely relevant in the decreased fearfulness observed in the adult offspring of high-LG mothers.

A critical issue is that of relating the epigenetic modifications at specific DNA regions to function. The presence of a methyl group on the 5′ CpG of the NGFI-A binding site is functionally related to glucocorticoid receptor gene expression in adult animals. In vitro studies revealed that the methylation of the 5′CpG site reduced the ability of NGFI-A to bind to the exon $1_7$ promoter and activate glucocorticoid receptor transcription (Weaver et al. 2007). These findings are consistent with the model described above, whereby DNA methylation impedes transcription factor binding and thus the activation of gene expression.

In contrast to the situation with neonates, there is no difference in NGFI-A expression as a function of maternal care among adult animals. Hippocampal levels of NGFI-A are comparable in the adult offspring of high- and low-LG mothers. However, the altered methylation of the exon $1_7$ promoter suggests differences in the ability of NGFI-A to access its binding site on the exon $1_7$ promoter. Chromatin-immunoprecipitation assays revealed increased NGFI-A association with the exon $1_7$ promoter in hippocampi from adult offspring of high- compared to low-LG mothers (Weaver et al. 2004, 2005; Hellstrom et al. 2012). This difference occurred despite the comparable levels of NGFI-A. These findings show that there is more

NGFI-A associated with the exon $1_7$ promoter in hippocampal neurons of adult animals reared by high- compared with low-LG mothers.

The maternal effect on the epigenetic state of the exon $1_7$ promoter to the changes in glucocorticoid receptor expression is linked to HPA responses to stress. CpG methylation dampened transcription factor binding through the recruitment of repressor complexes that include HDACs. A study (Weaver et al. 2004) examined the effects of directly blocking the actions of the HDACs in the adult offspring of high- and low-LG mothers by directly infusing the HDAC inhibitor trichostatin A (TSA) into the hippocampus daily for four consecutive days. The treatment with the HDAC inhibitor produced a series of predictable results that reflected a cause-effect relation between DNA methylation and gene expression. As expected, HDAC blockade eliminated the differences in the acetylation of the histone tails (open chromatin) of the exon $1_7$ promoter in hippocampal samples from high- and low-LG mothers. Second, the increased histone acetylation of the exon $1_7$ promoter in the offspring of low-LG mothers was associated with an increase in the binding of NGFI- A to the exon $1_7$ promoter in the offspring of low-LG mothers, eliminating the maternal effect on NGFI-A binding to the exon $1_7$ promoter. Comparable levels of NGFI-A binding to the exon $1_7$ promoter then eliminated the maternal effect on hippocampal glucocorticoid receptor expression, such that glucocorticoid receptor levels in the adult offspring of low-LG mothers treated with the HDAC inhibitor were comparable to those in animals reared by high-LG mothers. Most importantly, the infusion of the HDAC inhibitor reversed the differences in the HPA response to stress.

HDAC infusion into the hippocampus increased NGFI-A binding to the exon $1_7$ promoter in the adult offspring of low-LG mothers and decreased the level of methylation of the 5′CpG site on the exon $1_7$ promoter. Another study (Weaver et al. 2005) showed that the reverse pattern of results could be obtained in response to the infusion of methionine into the hippocampus. The methionine infusion produced greater methylation of the 5′CpG in the offspring of high-LG mothers, decreased NGFI-A binding and GR expression, and increased HPA responses to stress (Weaver et al. 2005).

Although these studies employ rather crude pharmacological manipulations, the results are critical, as they suggest that fully mature neurons in an adult animal express the necessary enzymatic machinery to demethylate or remethylate DNA. The importance of this plasticity at the level of DNA methylation has been revealed in subsequent studies of cognition (see below), which suggest that dynamic modification of DNA methylation in critical neuronal populations in adult animals is involved in specific forms of learning and memory.

## 3 Implications for Mental Health

There is now enthusiasm within the neuroscience community for epigenetic models of the effects of early experience, synaptic plasticity, and neural function. The hypothesis underlying this approach considers epigenetic effects on gene

expression to be candidate mechanisms for the effects of environmental signals on the future behavior of the organism. This hypothesis is particularly attractive for those examining the sustained effects of early experience or of chronic, biologically relevant events in adulthood (e.g., environmental enrichment, chronic stress) on gene expression and neural function. Mature neurons undergo considerable changes in phenotype and are therefore an ideal cell population for epigenetic regulation. Nevertheless, there are constraints on the influence of epigenetic marks. For example, the effects of DNA methylation on gene expression are influenced by the organization of the relevant genomic region. CpG density appears to influence the effect of DNA methylation on gene expression (Weber et al. 2007). Moreover, much of the DNA within a cell is packed tightly in heterochromatin (Fraser and Bickmore 2007) and is probably inaccessible to environmentally induced chromatin remodeling signals. Indeed, intracerebral infusion of an HDAC inhibitor produced a significant change in the transcription of $<2$ % of the hippocampal transcriptome in the rat (Weaver et al. 2006). It is likely that there is a pool of genes in a constitutively euchromatic state that retain the capacity for dynamic environmental regulation through epigenetic mechanisms.

We suggest that these findings provide researchers with a renewed appreciation of the environmental regulation of cellular activity, with implications for the study of psychopathology. There is emerging evidence for alterations in gene expression associated with DNA methylation in psychiatric illness. Cortical dysfunction in schizophrenia is associated with changes in GABAergic circuitry (Benes and Berretta 2001). This effect is associated with a decrease in the expression of the GAD1 gene that encodes for a specific form of glutamic acid decarboxylase (GAD67), one of two key enzymes for GABA synthesis in cortical interneurons. There is compelling evidence for the decreased expression of $GAD_{67}$ in cortical tissues from schizophrenic patients (Akbarian and Huang 2006; Costa et al. 2004). The dysregulated $GAD_{67}$ expression in chandelier GABA neurons is thought to result in disruption of synchronized cortical activity and impairment of executive functions in schizophrenia subjects (Lewis et al. 2005). Likewise, allelic variation in GAD1 is associated with schizophrenia (Straub et al. 2007).

In addition to $GAD_{67}$, there is a decrease in cortical expression of reelin in schizophrenic brains (Eastwood and Harrison 2003); reelin is closely associated with synaptic plasticity. The same GABAergic neurons in the schizophrenic brain that express reelin and $GAD_{67}$ exhibit an increase in DNA methyltransferases 1 (DNMT1; Veldic et al. 2004). The promoter for the reelin gene shows increased methylation in the brains of patients with schizophrenia compared with control subjects (Abdolmaleky et al. 2005; Grayson et al. 2005). Kundakovic et al. (2007) showed that the inhibition of DNMT1 in neuronal cell lines resulted in the increased expression of both reelin and GAD67. The increase in gene expression was associated with a decreased association of MeCP2, further suggesting that these differences are associated with alterations in DNA methylation. Recall that maternal care directly alters DNA methylation of the $GAD_{67}$ promoter in the rat (Zhang et al., 2010). This effect is associated with a decrease in DNMT1 expression and reduced MeCP2 association with the GAD1 promoter.

The rodent studies described above suggest that the early social environment, specifically parent–offspring interactions, can stably regulate transcriptional activity through epigenetic mechanisms. Recent studies (McGowan et al. 2009) have attempted to translate this hypothesis to humans. DNA was extracted from hippocampal samples obtained from victims of suicide or from individuals who had died suddenly from other causes (auto accidents, heart attacks, etc.). The samples were obtained from the Québec Suicide Brain Bank, which conducts forensic phenotyping that includes a validated assessment of psychiatric status and developmental history (e.g., McGirr et al. 2008). The studies examined the methylation status of the exon $1_F$ promoter of the glucocorticoid receptor, which shares about 70 % sequence overlap with the exon $1_7$ promoter in the rat and is highly expressed in the human hippocampus (Turner and Muller 2005). The results showed increased DNA methylation of the exon $1_F$ promoter in hippocampal samples from suicide victims compared with controls, but only if suicide was accompanied with a developmental history of child maltreatment. Child maltreatment, independent of psychiatric state, predicted the DNA methylation status of the exon $1_F$ promoter. As in the previous rodent studies, the methylation state of the exon $1_F$ promoter also determined the ability of NGFI-A to bind to the promoter and activate gene transcription.

Although such studies are obviously correlational and limited by postmortem approaches, the results are nevertheless consistent with the hypothesis that variations in parental care can modify the epigenetic state of selected sites of the human genome. Moreover, the findings are also consistent with studies that link childhood abuse to individual differences in stress responses (Heim et al. 2000). Childhood abuse is associated with an increase in pituitary ACTH responses to stress among individuals with or without concurrent major depression. These findings are particularly relevant, since pituitary ACTH directly reflects central activation of the HPA stress response, and hippocampal glucocorticoid receptor activation dampens HPA activity. The findings in humans are consistent with the rodent studies cited above investigating epigenetic regulation of the glucocorticoid receptor gene and with the hypothesis that early life events can alter the epigenetic state of relevant genomic regions, the expression of which may contribute to individual differences in the risk for psychopathology (Holsboer 2000; Neigh and Nemeroff 2006; Schatzberg et al. 1985).

Certain limitations need to be considered as we integrate epigenetics into the study of psychopathology. The study of epigenetic mechanisms in humans is complicated by the fact that epigenetic marks are often tissue-specific. The process of cell differentiation involves epigenetic regulation and implies that the epigenetic marks vary from cell type to cell type. Indeed, there is considerable variation in epigenetic marks from one brain region to another, perhaps even more so than variation within the same brain region across individuals (Ladd-Acosta et al. 2007). Brain samples are for the most part beyond direct examination in the living individual at the level of molecular analysis, which often leaves us with measures of DNA extracted from blood or saliva and with the question of whether the epigenetic marks within such samples actually reflect those within the relevant

neuronal population. Thus, for the time being, advances in the study of "neuroepigenetics" will rely heavily on relevant models with nonhuman species as well as complementary studies of samples from postmortem human brains. We await resolution of the question regarding the use of potentially viable 'proxy' samples, such as those from blood or buccal sampling. One option for those interested in examining the regulation of epigenetic states across the genome by the social environment lies in the area of psychoneuroimmunology. This field of study examines the influence of the social environment and accompanying mental states on immune function. An advantage, of course, is that the cells of the immune system are more accessible and relate to important health states.

## 4 Environmental Epigenetics and Immune Function

The environment also plays an important role in how the immune system responds to pathogens across the life span. The study of epigenetic mechanisms in the modulation of immune function is a burgeoning field and is beginning to demonstrate how the environment may have a long term impact on an organism's risk for several diseases, including cancer, cardiovascular disease, autoimmune diseases, allergy, asthma and depression through epigenetic modifications that influence the gene expression of immune-related genes (Mathews and Janusek 2011). Further basic research is demonstrating the importance of epigenetic mechanisms in the development and differentiation of immune cell populations involved in the adaptive immune response to infection (Janson and Winqvist 2011). Several environmental events, especially in prenatal and early in postnatal life, lead to epigenetic modifications of genes involved in mediating effector immune responses. This process in turn leads to individual differences in the propensity to eradicate pathogens and in the likelihood of developing immune-related diseases (Martino et al. 2011).

## 5 Epigenetic Modifications Modulate the Differentiation of Immune Cell Subtypes

Immune cells that form part of the adaptive immune response to infection undergo a series of epigenetic modifications involving changes in the structure of chromatin and various covalent modifications in order for multipotent progenitor cells to give rise to differentiated daughter cells with a more restricted set of capacities across the life span (Reiner 2005; Cuddapah et al. 2010; Martino and Prescott 2010). Hence different T and B cell subsets develop that can target specific bacteria, viruses, parasites and tumors (Janson and Winqvist 2011). Epigenetic modifications to the genome also allow dividing immune cells to imprint various signaling events that happened earlier in their development, imbuing them with a form of immunological "memory" (Reiner 2005; Janson and Winqvist 2011; Agarwal et al., 2009).

B and T lymphocytes develop in the bone marrow and thymus, respectively. Within the thymus, newly formed T cells undergo epigenetic modifications when the T cell receptor (TCR) is activated. This activation involves the recruitment of Protein kinase D1 (PKD1), which then interacts with HDAC7, a class II deacetylase and phosphorylates three serines (Ser155, Ser318, and Ser448) at HDAC7's N terminus, leading to the export of HDAC7 from the nucleus and thus increasing gene expression within newly activated T cells (Parra et al. 2005.)

Upon the exit of newly formed T and B cells from the thymus and bone marrow, the cells are still considered naïve until they encounter their respective antigens. Upon antigen presentation, they undergo further division and differentiation where daughter cells show marked changes in the expression of key regulatory cytokines as compared to the original progenitor cells (Reiner 2005). To exemplify, helper T cells, which form part of the adaptive immune response to infection, can become either Th1 cells or Th2 cells upon encountering a pathogen. Th1 cells mediate the immune responses of phagocytes that neutralize intracellular pathogens, and they are important in the elicitation of host defenses against cancer. Th2 cells use humoral defenses to eradicate extracellular pathogens. Th1 and Th2 cells differ markedly in their cytokine gene expression profile. Th1 cells express IFN-gamma but not interleukin (Il) 4, 13 or 5, whereas Th2 cells express Il 4, 5 and 13, which stimulate B-cell antibody production but do not secrete IFN-gamma (Abbas et al. 1996). This differentiation process from a naïve helper T cell to a mature Th1 or Th2 cell occurs over the process of multiple cell divisions. Recent evidence links the involvement of chromatin configuration and re-configuration in this process of T cell effector differentiation. How a cell becomes one subtype over another is dependent on the cytokine milieu at the site of antigen presentation. When the surrounding cytokines bind to their appropriate cytokine-specific receptors on the pluripotent immune cell surface, an intracellular signaling cascade is initiated that culminates in the binding of specific transcription factors to key genomic cytokine promoters. The nuclear translocation of these transcription factors is involved in the remodeling of chromatin at these promoter regions (Wilson et al., 2005).

DNA methylation, demethylation and DNAse I hypersensitivity sites are activated and involved in the coordinated expression and repression of the effector cytokine genes, Il4 and Ifng in naïve T cells. At this stage their expression is not completely transcriptionally silent. When a progenitor T cell becomes activated by a pathogen, there is still submaxinal transcriptional activity of both genes. As the cells continue to mature, more restricted patterns of transcription occur, involving acetylation of the loci encoding the desired cytokines. Activation of a naïve helper T cell also involves the expression of one of two master regulatory transcription factors, GATA-3 and T-bet, which regulate Th1 and Th2 differentiation. They are also implicated in chromatin remodeling of their putative target genes, Ifng and Il4. Further the co-regulated expression of IL-4, IL-5 and IL-13, which often occurs in mature Th2 cells, may be due to the fact that, while these genes are located at some distance from each other on the genome, they are looped together around a locus control region residing in the rad50 gene body (Reiner 2005).

The ratio of different T and B cell effector populations that arise from naïve pluripotent immune cells is critical in maintaining proper immunohomeostasis. An imbalance between Th1 and Th2 immune cell populations has been linked to the development of several immune-related diseases. A shift in the balance towards greater Th1 cell production has been linked to the development of autoimmune diseases, whereas the development of allergies is associated with greater Th2 immune cell production. A dysregulation in the epigenetic mechanisms that control Th1 and Th2 cell differentiation is thus involved in immune-related disorders. The development of allergies and asthma has been associated with events during prenatal development. The adaptive immune response to infection under Th1 cell control only matures during early childhood, at which point a reduction or silencing of Th2 cell function should occur. In the absence of silencing of heightened Th2 humoral immune responses, allergies and asthma develop (Bousquet et al. 2004). Hence critical experiences, including exposure to psychosocial stress during prenatal and early postnatal development, can have a long-term influence on the propensity to develop immune-related diseases in later life, due to an alteration in the balance between Th1 and Th2 cell differentiation through modifications in epigenetic marks on the promoters of cytokine and immune receptor genes.

One of the main characteristics of the adaptive immune response to infection is that its effector immune cells can develop immunological memory after an encounter with a specific pathogen. This process is directly analagous to that occurring in neurons (Day and Sweatt 2010). For example, after exposure to a specific antigen, memory CD8 + T cells can rapidly set up an adaptive immune response when re-exposed to the same antigen. In little time, high levels of cytokines are released from the memory T cells, target cells are quickly eradicated and many secondary effector cells are produced following re-exposure to the pathogen. The ability of memory CD8 + T cells to effectively combat the same pathogen upon re-exposure is dependent on the presence of CD4+ T cells. The presence of CD4 + T cells is important in leading to chromatin remodeling of the newly formed CD8 + T memory cells. Immunological memory is dependent on active gene expression that involves increased histone acetylation of many immune genes in CD8 + T memory cells. In the absence of CD4+ T cells, the newly formed CD8 + T memory cells show hypoacetylation on the promoters of several immune-related genes. The loss of CD4 + T cell involvement can be rescued using an HDAC inhibitor to restore histone acetylation to the CD8+ T cell genes upon antigen exposure. Hence the development of immunological memory in CD8+ T cells is dependent upon epigenetic modifications (Northrop et al. 2008).

## 6 Epigenetics and Psychoneuroimmunology

Different forms of psychosocial stress have also been associated with the epigenetic modification of immune-related genes in both animal models and humans. Chronic social defeat stress is a paradigm often used to elicit a depressive-like phenotype in rodents. Epigenetic modifications, including decreased H3 methylation followed by

an increase in the binding of the transcription factor phospho-CREB to several immune-related gene promoters, occur following chronic social defeat stress. This finding is interesting in light of the positive correlation often associated between depression, sickness and the inflammatory response (Dantzer et al. 2008). A decrease in the methylation profile of several immune-related genes has been associated with immune activation and possibly a greater inflammatory response in individuals that developed post-traumatic stress disorder (PTSD) following exposure to a traumatic experience compared to control subjects (Segman et al. 2005; Zieker et al. 2007). In turn, patients with PTSD showed a greater antibody titer response to cytomegalovirus, a latent herpes virus (Wrona 2006). This study was cross-sectional in design, so it is not possible to ascertain if these differences in methylation profile emerged following exposure to the traumatic event or if they existed prior to the development of PTSD (Mathews and Janusek 2011).

Besides psychosocial stress, long-term exposure to ethanol has been shown to have adverse effects on emotionality and cognition, leading to increased neuroinflammation in the frontal cortex, striatum and the hippocampus. Notably, some of these alcohol-mediated effects appear to be mediated through Toll-like receptor 4 (TLR4), a receptor important in the mediation of the innate immune response to infection. While wild-type mice exposed to chronic ethanol show behavioral impairments associated with reduced H4 histone acetylation and histone acetyltransferase activity in the hippocampus, striatum and frontal cortex, TLR4 null mutant mice did not show any behavioral impairments in emotionality or cognition; neither did they have a reduced acetylation profile in these same brain areas following chronic alcohol administration (Pascual et al. 2011).

Further evidence that the environment can lead to epigenetic changes in immune-related genes comes from the study of monozygotic twins (MT). Peripheral blood lymphocytes from young and older MT pairs were collected for the study of the global methylation pattern as well as the histone 3 and histone 4 acetylation patterns of the genes in these lymphocytes. As the twins aged, the epigenetic fingerprint diversified further between pairs of twins and was the most divergent for twin pairs that spent a greater portion of their lives apart (Fraga et al. 2005).

The development of several immune-related diseases has also been linked to epigenetic modifications, including increased histone acetylation, decreased DNA methylation and/or modifications to non-coding RNA expression in different immune cell populations. These epigenetic modifications are associated with the development of rheumatoid arthritis, systemic lupus erythematosus (SLE; Trenkmann et al. 2010; Martino and Prescott 2010), multiple sclerosis (Lincoln and Cook 2009), type 1 diabetes, celiac disease, idiopathic thrombocytopenia (Brooks et al. 2010), asthma and allergy (Martino and Prescott 2010; Handel et al. 2010; Mathews and Janusek 2011). Further, psychosocial stress exacerbates many of these immune-related disorders and may play a causative role in the development of these diseases in biologically vulnerable individuals through epigenetic modifications of immune-related genes (Mathews and Janusek 2011).

SLE involves an increase in autoreactive white blood cells and the development of antibodies that attack self-antigens. SLE patients have a reduced methylation of genes in DNA from CD4+ lymphocytes. A greater degree of hypomethylation of genes in this immune cell population is correlated with greater symptomatology in SLE patients (Richardson et al. 1990). Several autoreactivity-promoting genes, including those for IL-4, IL-6, perforin, immunoglobubin (Ig)-like receptor, and B-cell costimulatory molecules including CD70, CD6 and CD154, are thus overexpressed in SLE patients (Zhao et al. 2010; Lu et al. 2005, 2007; Liu et al. 2009; Lal and Bromberg 2009). Several CpG islands are also hypomethylated in B cell subtypes from SLE patients compared to control subjects (Garaud et al. 2009). SLE is also characterized by a reduction in the number of functioning regulatory T cells that suppress effector T lymphocytes, B lymphocytes and antigen-presenting cells. The transcription factor, FOXp3, is the master regulator of Treg development. However, in SLE patients, many T cells express FOXp3 through hypomethylation of the FOXp3 gene promoter but do not function as Treg cells, thus leading to increased activity by immune effector cells (Lal and Bromberg 2009). Further, the increased incidence of autoimmune diseases among women has been linked with demethylation of the inactivated X chromosome in CD4+ T cells from these patients, thus increasing their exposure to immune-related gene expression (Lu et al. 2007). Patients with SLE have higher IL-6 release from autoreactive B cells compared to control subjects. When IL-6 is antagonized using an IL-6 antiserum, the increased spontaneous production of autoreactive antibodies is reduced and DNA methylation is restored in B cells from SLE patients (Lu et al. 2007). Thus blocking the autocrine IL-6 cytokine loop could be used as a therapeutic intervention for SLE.

The increased exposure to glucocorticoids following psychosocial stress could be mediating some of these differences in immune-related function. While psychosocial stress is linked to immune activation in some immune cell populations, including those involved in the inflammatory response, psychosocial stress is also associated with a decrease in the activation of natural killer (NK) cells that are involved in the eradication of tumor cells and several pathogens. Treatment of the human NK cell line with dexamethasone, a synthetic glucocorticoid, leads to a significant reduction in NK cell activity through a reduced capacity for the NK cells to bind to tumor targets and to release perforin and granzyme B, which are involved in the eradication of tumor cells. Further, the expression of several pro-inflammatory cytokines, including IL-6, TNF-alpha and IFN gamma, was reduced both constitutively and following stimulation. These changes were linked to epigenetic modifications, including a reduction in global histone acetylation, H4K8 acetylation and in the accessibility of the perforin, interferon gamma and granzyme B promoter regions. Histone acetylation was increased following treatment with an HDAC inhibitor, which led to the recovery of NK cell activity through restoration of higher cytokine and cytolytic protein production (Krukowski et al., 2011).

Epigenetics plays a fundamental role in the development of the adaptive immune response through the control of both T and B cell differentiation and in the development of immunological memory. Epigenetics is also an important mechanism to explain how environmental events such as exposure to psychosocial stress

can impact the functioning of the immune response, leading to individual differences in the development of immune-related diseases and in the propensity to develop illness. The merging of epigenetics with psychoneuroimmunology, while still in its infancy, has already furthered our understanding of how the environment can have a lasting impact on our risk for disease through modifications at the level of the epigenome. Cells of the immune system, like those in the brain, undergo extensive, post-mitotic phenotypic modifications. Moreover, various psychiatric illnesses associate with alterations in immune function, including PTSD and depression.

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# Epigenetic Inheritance in Mammals

**Johannes Bohacek and Isabelle M. Mansuy**

**Abstract** The epigenetic state of a cell is determined by epigenome that represents the ensemble of modifications occurring at the chromatin. These modifications involve the specific marking of DNA and protein histones, which provides a potent and dynamic mode of regulation of the genome without changing the DNA sequence itself. In mammals, the epigenome was previously thought to be fully erased after fertilization and then partly reestablished for further cellular development and differentiation. It is now recognized that erasure is not complete and that some epigenetic marks are maintained and passed to the offspring. Such maintenance is postulated to provide a unique mode of epigenetic inheritance, but this proposal has been the subject of much debate. Now, there is increasing evidence to show that it does occur in rodents and humans. This review discusses some of this evidence and describes examples of epigenomic transmission of traits influenced by environmental factors such as diet, toxic agents, stress and environmental enrichment across generations.

## 1 Introduction

It is commonly accepted that heritable information is transmitted from parents to offspring through genomic DNA and that the traits of the offspring are determined by the combination of DNA sequences from both parents. However, recent work in the field of epigenetics has proposed that the genome is not the only substrate for trait inheritance in mammals, and that the epigenome contributes a significant part. Since the epigenome can be modulated by environmental factors such as diet, maternal care, early-life experiences, chemicals, aging, etc., epigenetics also provides a unique

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means to introduce phenotypic variation that can persist throughout an individual's life time. These variations may possibly be transmitted to the following generations if the epigenetic changes occur in germ cells (Curley et al. 2011a, b; Daxinger and Whitelaw 2010; Feng et al. 2010; Franklin and Mansuy 2010a, b; Hochberg et al. 2011; Skinner et al. 2010). Therefore, the epigenome provides a unique interface between the genome and external factors and represents a potential mediator of adaptive processes across evolution. This review discusses some of the examples of transgenerational epigenetic inheritance that have been reported in human and animal studies, and attempts to provide evidence for the impact of environmental factors on individuals across several generations.

## 2 Diet

Clinical and epidemiological studies have demonstrated that nutrition and food availability have a strong and persistent influence on well-being and can have continuing effects on health across generations. Both an excess and a shortage of food and nutrients have an effect. In humans, the amount of food supply during the slow-growth period, a period that precedes the prepubertal peak in growth, has been associated with mortality risk in following generations (Kaati et al. 2007; Pembrey 2010; Pembrey et al. 2006). Overeating in individuals during this sensitive period can reduce life expectancy in the grandchildren of these individuals (Bygren et al. 2001), which is postulated to be due, in part, to an increased risk for the development of cardiovascular disease and diabetes (Kaati et al. 2007). This risk was reported to affect both women and men, although transmission was gender-specific and food supply in paternal grandmothers was associated with granddaughters' mortality risk, whereas food supply in paternal grandfathers influenced grandsons' mortality risk (Kaati et al. 2007; Pembrey et al. 2006). Diet also has a strong impact on birth weight that can be seen across several generations. In Western Holland during World War II, women who were severely food deprived during the last trimester of pregnancy due to an embargo on food supplies delivered babies with drastically reduced birth weights (Susser and Stein 1994). This negative effect on weight was also observed in babies from subsequent generations, despite no further dietary restriction during pregnancy or later development, suggesting a persistent and transmissible effect.

As with humans, rodents' health and physiological functions are also affected by diet. In rats, feeding of a low-protein diet (8-11 % instead of 20 %) prior to and throughout gestation was shown to have an effect on metabolic processes on the offspring (Benyshek et al. 2006; Carone et al. 2010). The F1 offspring of parents fed a low-protein diet had reduced birth weights and lower insulin secretion, an effect that is transmitted to the following F2 and F3 generations. In these offspring, brain and body weight are lower, maturation is slower and insulin resistance rate is increased. In F3 animals, despite receiving a normal diet throughout life, and despite being born to F2 females that were fed a normal diet, glucose resting

level is higher in females, and the level of insulin is increased in males, suggesting a persistent effect of diet across several generations. High-fat diet exposure of females was also shown to increase body length and reduce insulin sensitivity in their offspring two generations down from the initial exposure (Dunn and Bale 2009). These abnormalities were transmitted through both mothers and fathers, and the effect was amplified when female and male offspring of maternal high-fat diet exposure were crossbred (Dunn and Bale 2009). A chronic high-fat diet in male rats was also shown to program β-cell dysfunctions, induce higher body weight, adiposity, impaired glucose tolerance and insulin sensitivity in female offspring (Ng et al. 2010). The mechanisms underlying the effects of diet are not understood, but initial evidence has pointed to epigenetic alterations involving DNA methylation. In the brain of the offspring of parents fed a high-fat diet across two generations, DNA methylation is altered at the gonadotrophin hormone secretagogue receptor (GHSR) promoter (Dunn and Bale 2009). More research will be needed in the future to determine the contribution of such epigenetic mechanisms and will help better understand the long-term influence of diet on the epigenome (McKay and Mathers 2011).

## 3 Environmental Toxicants

Environmental toxins also have persistent transgenerational effects on humans and animals. One of the most dramatic examples is diethylstilbestrol, a synthetic non steroidal estrogen prescribed in the 1970s to prevent miscarriage in women. Diethylstilbestrol was efficient in increasing the rate of term pregnancy, but it had severely detrimental and persistent side effects. It disturbed developmental processes and increased the risk of breast cancer and adenocarcinoma in baby girls (Palmer et al. 2006). The risk of cancer was high in the girls exposed to the drug during embryogenesis but was also transmitted to the following generation. Thus, women whose maternal grandmothers received treatment during early pregnancy had an increased risk of developing ovarian cancer (Titus-Ernstoff et al. 2008), and cases of small cell carcinoma in the ovary were also reported (Blatt et al. 2003). This transgenerational effect of diethylstilbestrol was also observed in mice in which, as in humans, perinatal exposure to diethylstilbestrol altered uterine development and induced uterine cancer in F1 and F2 offspring. This effect was suggested to result from aberrant DNA methylation in a gene that controls uterine development (homeobox gene HOXA10) and in uterine cancer genes (Bromer et al. 2009; Li et al. 2003; Newbold et al. 2006; Walker and Haven 1997).

Another striking instance of a cross-generational effect was reported for the endocrine disruptor vinclozolin, an anti-androgenic compound used as a fungicide in fruit crops. In the rat, a single exposure to vinclozolin during the critical period of embryonic development increased the risk of infertility, tumor formation and kidney disease and induced immune anomalies in exposed individuals and their offspring across four generations (Anway et al. 2005; Jirtle and Skinner 2007). It also led to anxiety behaviors that were transmitted. These severe effects were

associated with aberrant DNA methylation in multiple genes in the sperm across generations (Anway et al. 2005).

Epigenome-wide DNA methylation analyses in sperm identified 16 genes with altered DNA methylation in the F3 generation offspring of animals exposed to vinclozolin (Guerrero-Bosagna et al. 2010). These and similar alterations observed in imprinted genes, indicate a persistent effect of vinclozolin on the epigenome across generations, which is thought to contribute to the maintenance of its detrimental impact (Stouder and Paoloni-Giacobino 2010). Further, a consensus DNA sequence that is more prevalent in the promoter of the altered genes as well as in imprinted genes was also identified (Stouder and Paoloni-Giacobino 2010), suggesting that specific sequences may render certain regions of the genome more sensitive to environmental factors and/or perhaps more resistant to the erasure of epigenetic marks during developmental reprogramming events.

## 4 Trauma in Early Life

The quality of environmental conditions experienced early in life is an important factor for the development of proper behavioral responses. When early life conditions are poor or perturbed by trauma, abuse or violence, they can have a detrimental impact on individuals throughout their adult life, but also affect their offspring. In humans, maltreatment and childhood trauma are known to increase the risk of depression and anxiety disorders in adult individuals (Iversen et al. 2007). The high level of transmission of such disorders has been further associated with the quality of parenting and can be predicted from personal factors, such as temperament (Dierker et al. 1999; Manassis et al. 1995; Merikangas et al. 1998; Shamir-Essakow et al. 2005; Weissman et al. 1984). Epidemiological studies thus revealed that the attachment of a child strongly influences her/his ability to form appropriate peer and social relationships, perform well in school, and have normal behavioral responses (Harper 2005; Sroufe 2002).

In humans and experimental animals, persistent disorders resulting from maltreatment early in life have been associated with alterations in DNA methylation. Methylation in the neuron-specific glucocorticoid receptor promoter was reported to be higher in the brains of suicide victims who experienced childhood abuse than in victims with no abuse, and the increased methylation was associated with decreased glucocorticoid receptor expression (McGowan et al. 2008). Likewise in rodents, poor maternal care was associated with behavioral impairments and anxiety and with alterations in the profile of DNA methylation in the brain of the offspring when adult (Weaver et al. 2007).

The impact of early trauma across generations has, however, only recently been examined experimentally. Our own work in mice demonstrated that traumatic experiences early in life can have a negative impact across multiple generations (Franklin et al. 2010; Weiss et al. 2011). We developed a mouse model of early life stress based on unpredictable maternal separation and maternal stress (MSUS). In this model, newborn pups are separated from their mother unpredictably for three

hours every day during the first two weeks after birth, and their mother is exposed to additional stress unpredictably during the separation period. This novel model is instrumental in that it captures the negative aspects of early childhood trauma, which in humans constitute major risk factors for the development of mood and personality disorders. We observed that MSUS treatment led to depressive-like and antisocial behaviors in the offspring when adult. Strikingly, the second-generation offspring, produced by breeding F1 animals to a wild-type naïve partner, exhibited behavioral alterations similar to their F1 parent. Behavioral inheritance was observed through both males (Franklin et al. 2010) and females (Weiss et al. 2011), indicating a consistent effect regardless of sex. Importantly, in the maternal line, the behavioral alterations could not be reversed by cross-fostering, suggesting that transmission did not involve maternal care. This finding is also consistent with the fact that transmission also occurred through males, which were only present for mating and did not have any contact with the offspring. Further, transmission through males was demonstrated to occur across two successive generations, and animals from the third generation (F3) had behavioral alterations similar to the animals directly subjected to stress. This is strong evidence that non-genomic mechanisms are at play, since the germ cell from F2 males giving rise to F3 animals was never directly exposed to stress. Initial molecular analyses provided insight into some of the genes that are altered by early stress. When examined in the sperm of F1 males, DNA methylation was found to be changed in the promoter-associated CpG islands of several genes, in particular, methyl-CpG binding protein 2 (MeCP2), cannabinoid receptor 1 (CB1), and corticotrophin releasing factor receptor 2 (CRFR2). Importantly, similar alterations were also observed in the brains of the F2 progeny and were paralleled by changes in gene expression. Thus, since the germline represents the only direct biological link between generations, these data provide strong evidence for a relationship between altered DNA methylation and transmission of behavioral traits across generations induced by early life stress (Franklin and Mansuy 2010a).

## 5 Enriched Environment

In contrast to early trauma and stress, an enriched environment can have positive effects on behavioral responses and cognitive development that, in some cases, can be transmitted across generations (Arai et al. 2009). Mice exposed to enriched environmental conditions, including living in a social group in a large cage and having access to toys, novel objects, and a running wheel, etc, in early life have enhanced memory performance and increased synaptic plasticity when adult. Improved performance can also be observed in the offspring and be transmitted through females, not males.

Importantly, transmission was shown to be independent of maternal care, since the effect persisted even after cross-fostering. These results therefore suggest the contribution of epigenetic mechanisms to the positive impact of enriched environment on cognitive functions.

## 6 Conclusions

A growing number of reports have described transmission of behavioral traits, physiological alterations or disease states induced by environmental factors across multiple generations. In the most striking of these reports, transmission was shown down to the third generation, which provides strong evidence for non-genomic inheritance since third generation individuals arise from germ cells that were never directly exposed to the manipulation or the drug. Initial evidence for potential mechanisms has suggested that DNA methylation in germ cells may contribute to the observed epigenetic inheritance. Since DNA methylation in the germline is sensitive to multiple environmental factors and can be modulated during development, its vulnerability may underlie the occurrence of persistent changes across generations. But other mechanisms besides DNA methylation, including short non-coding RNAs, histone PTMs or DNA repair, may also contribute to epigenetic inheritance (Arnaud 2010; Eun et al. 2010).

Conceptually, it is important to consider why such mechanisms for transgenerational transmission exist. One great advantage of epigenetic inheritance over classical inheritance is that it allows rapid and selective adaptation to novel environmental challenges and the maintenance and transmission of such adaptive process from one generation to the next (Harper 2005). This adaptation favors the appropriate preparation of the offspring to altered environmental conditions. For instance, increased insulin sensitivity in the offspring of females exposed to scarce food supply might lead to more adipogenesis, which raises nutritional reserves and protects the body from potential future starvation (Godfrey et al. 2010). It makes the offspring better equipped than its parents to deal with unfavorable conditions with limited food resources. Importantly, such adaptation can be reversed in following generations if the environment becomes more favorable or is markedly different from the "anticipated environment". However, if not reversed, it may leave the individual maladapted and render physiological responses inappropriate. For instance, elevated insulin sensitivity in an environment with abundant food resources may lead to obesity and diabetes. Likewise, an altered response to highly stressful life conditions may become maladaptive in a normal and balanced environment. It will be important to address these considerations in the future. Finally, the extent to which transgenerational epigenetic inheritance occurs and the mechanisms involved will also need to be investigated. The development of powerful and sensitive genome- and epigenome-widescreening techniques will be instrumental and are is expected to yield fundamental new insights into the epigenetics of complex biological functions.

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# Control of Neuronal Gene Transcription and Behavior by the Epigenetic Suppressor Complex G9a/GLP

**Anne Schaefer**

**Abstract** Epigenetic control of cellular phenotypes is governed by numerous enzymes that contribute to post-synthetic modifications of DNA and associated histone proteins. These modifications facilitate the assembly of protein complexes that regulate gene expression in a highly orchestrated fashion. The importance of epigenetic regulators in brain development and function is supported by the strong association between mental retardation in humans and the aberrant structure or expression of various epigenetic regulators

Using conditional mutagenesis in mice, we found that the histone methyltransferase complex G9a/GLP (Ehmt1/Ehmt2 in humans), which controls gene expression via di-methylation of histone 3 on lysine 9 (H3K9me2), regulates cognition and complex behavior in mice. Postnatal neuron-specific loss of G9a/GLP in mice recapitulates key symptoms of a severe mental retardation syndrome in humans that is associated with the reduced expression of GLP/Ehmt1. In an attempt to unravel the mechanism of G9a/GLP regulated cognition and behavior, we will discuss the nature of gene expression changes associated with the loss of G9a/GLP.

## 1 Introduction

The adult brain consists of a large variety of neuronal cells types that comprise highly specialized brain areas and perform specific functions. While normal brain function depends on the coordinated activity of highly specialized neurons, individual neuronal activities require coordinated function of neuron-expressed gene

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networks. Similar to other cells in the organism, differentiated neurons acquire stable patterns of gene expression that are maintained over the neuronal lifetime and define the neuron's identity as well as its functional specificity (Ooi and Wood 2008; Qiu and Ghosh 2008). Non-neuronal and other neuronal cell type-specific genes must remain stably suppressed to ensure unaltered neuronal function. On the other hand, to respond adequately to the rapidly changing environment, it is essential for differentiated neurons to maintain a certain degree of flexibility. To ensure an adequate behavioral response to environmental stimuli, numerous neuronal genes are required to undergo significant changes in their activity. Finally, the genetic nature of memory (Borrelli et al. 2008; Kandel 2001) implies the existence of mechanisms that hard-wire external signals into genetic changes that account for retrievable patterns of neuronal activity. This neuron-specific transcriptional networks are defined by the interplay between the expression of specific transcriptional regulators and the neuronal cell type-specific epigenome.

The epigenetic regulation of gene transcription depends on post-synthetic modifications of DNA and associated chromatin proteins. A single unit of chromatin is represented by four pairs of histone proteins and the DNA wrapped around them. The N-terminal portion of each histone is subjected to numerous post-translational modifications, including methylation, acetylation and phosphorylation (Berger 2007; Cheung et al. 2000; Crosio et al. 2003; Kouzarides 2007; Strahl and Allis 2000). Given the reversible nature of these modifications, it is very likely that, at any given moment in time, each nucleosome possesses a unique pattern of histone modifications, which, in turn, should lead to highly dynamic, individual nucleosome-specific recruitment of specific histone binding proteins that recognize the individual histone modifications and lead ultimately to activation or suppression of transcriptional activity. The negative control of gene expression is regulated by DNA methylation as well as methylation of histone H3 and H4 at lysine 9/27 or 20, respectively (H3K9me2/3, H3K9me3, H3K27me3 and H4K20me1/3; Bird 1986; Jenuwein and Allis 2001; Kouzarides 2007). These posttranslational histone modifications contribute to formation of chromatin structures that prevent gene activation and are essential for neuronal cell differentiation through suppression of cell lineage non-specific genes. We hypothesized that, in addition to maintaining lineage identity, epigenetic regulation of gene suppression contributes to formation of stable phenotypes of highly specialized neuronal cells in the various brain areas.

One of the major euchromatic suppressive histone modifications regulating cell differentiation and lineage commitment is H3K9me2 (Lehnertz et al. 2010; Rice et al. 2003; Tachibana et al. 2002; Wen et al. 2009). While genome-wide H3K9me2 analysis in embryonic stem (ES) cells revealed a very low abundance of H3K9me2, the commitment to a specific cell lineage is characterized by acquiring large, cell-type specific regions of high H3K9me2 abundance (Wen et al. 2009). The high abundance of H3K9me2 in differentiated cell types such as neurons or liver cells is functionally associated with suppression of non-lineage-specific gene expression. In neurons, more than 10 % of all protein encoding genes are found in very large, H3K9me2-enriched regions and most of these genes are non-neuronal genes Wen et al. 2009. Conversely, many genes that are neuron-specific, as well as genes that belong to the family of immediate early genes such as Jun, Fos, and Egr1, display relatively low levels of H3K9me2.

## 2 Loss of G9a/GLP in Postnatal Neurons Erases Euchromatic H3K9me2

To address to what extent H3K9me2 regulates neuronal identity and function in the adult brain, we generated mice with a postnatal, brain-specific ablation of euchromatic H3K9me2. The "erasure" of H3K9me2 has been achieved by conditional inactivation of the methyltransferase complex, G9a/GLP, which plays a key role in di-methylation of H3K9 (Sampath et al. 2007; Tachibana et al. 2001, 2002, 2005), in postnatal forebrain neurons. Functional inactivation of G9a and GLP in postnatal neurons was achieved by crossing mice in which the catalytic SET-domains containing exons of G9a and GLP are flanked by LoxP sites with mice that express Cre recombinase in postnatal forebrain neurons specifically (Camk2a-Cre: Zhang et al. 2004). In addition to Camk2a-Cre mice, we also used mice that enable conditional ablation of G9a and GLP selectively in dopamine 1 receptor- (Drd1) or dopamine 2 receptor- (Drd2) expressing neurons (Gong et al. 2007).

Immunohistochemical analysis of brain tissues from *Camk2a-Cre; GLP*$^{fl/fl}$ and *Camk2a-Cre; G9a*$^{fl/fl}$ mice showed a specific and drastic reduction in euchromatic H3K9me2 levels (Schaefer et al. 2009). The loss of euchromatic H3K9me2 upon ablation of either G9a or GLP in postnatal forebrain neurons confirmed the previously reported essential role for the intact G9a/GLP complex in euchromatic H3K9me2 (Peters et al. 2003; Sampath et al. 2007; Tachibana et al. 2001, 2002, 2005). In agreement with the previously reported selective role of G9a/GLP in formation of euchromatic H3K9me2 in various cell types (Peters et al. 2003; Sampath et al. 2007; Tachibana et al. 2001, 2002, 2005), the loss of G9a/GLP had no impact on neuronal heterochromatic H3K9me3 formation (Schaefer et al. 2009).

Deficiency in GLP or G9a and loss of euchromatic H3K9me2 in postnatal neurons had no apparent impact on the structural organization of the affected brain areas. Moreover, the neuronal cell type-specific morphological and basal electrophysiological properties remained unaltered (Schaefer et al. 2009). Preservation of neuronal survival, morphology, and electrophysiological properties rules out an essential role for G9a/GLP in vital cellular processes, a conclusion that is consistent with previously described results from G9a-deficient fibroblasts, cardiomyocytes and lymphocytes (Sampath et al. 2007; Shirato et al. 2009; Thomas et al. 2008).

## 3 Postnatal, Neuron-Specific Loss of GLP/G9a in Mice Reproduces key Signs of Severe Human Mental Retardation Syndrome

In humans, loss or genetic alterations of the subtelomeric region q34 of chromosome 9, which includes the GLP/Ehmt1 gene, are associated with a severe mental retardation syndrome (9q34 or Kleefstra syndrome; Chen and Garg 1999; Cormier-Daire et al. 2003; Kleefstra et al. 2005, 2009; Yatsenko et al. 2005). The potential causal role for GLP/Ehmt1 gene alterations in the human 9q34 syndrome has been

underscored by the identification of various patients with intragenic GLP/Ehmt1 mutations that include GLP micro-deletions, presence of premature termination codons, and an amino acid substitution in the catalytic SET domain that would affect the histone methyltransferase activity of GLP (Kleefstra et al. 2005, 2006, 2009). Clinically, the 9q34 syndrome is characterized by severe learning deficits, decline in goal-directed cognition and behavior, reduced social and emotional responsiveness, and reduced motor activity in children that can exacerbate to an apathy-like syndrome in adults.

In support of an important role for GLP in regulation of cognition and complex behaviors in humans, we found that mice with a postnatal forebrain-specific G9a/GLP deficiency display severe behavioral abnormalities (Schaefer et al. 2009). Starting around six to eight weeks of age, *Camk2a-Cre; GLP*$^{fl/fl}$ or *Camk2a-Cre; G9a*$^{fl/fl}$ mice show a diminished motor and explorative activity in the open field analysis. Notably, a similar reduction in motor activity and exploration can be observed in mice with a haploinsufficiency in *GLP*, a genetic condition similar to the human 9q34 syndrome patients (Balemans et al. 2010; Schaefer et al. 2009). Combined postnatal neuron deficiency of GLP and G9a does not potentiate the abnormal activity observed with a single enzyme deficiency, confirming the previously described essential role for the G9a/GLP heterodimer complex in H3K9me2 formation (Schaefer et al. 2009). The reduction in locomotor activity does not reflect a defect in motor function or balance; neither is it caused by an increased anxiety phenotype in the *Camk2a-Cre; GLP*$^{fl/fl}$ mice. On the contrary, mice with a postnatal forebrain-specific deficiency in GLP appear not to be aware of or interested in the potential danger of an open, unprotected environment that, in the wild, would make them easy prey for potential predators. Consistent with the lack of exploratory activity in a novel environment, *Camk2a-Cre; GLP*$^{fl/fl}$ mice do not display interest in novel objects or social interaction; neither do they respond to natural food rewards such as sucrose. These data indicate potential deficits in the brain motivation and reward system. Moreover, we found that the deficiency of GLP in postnatal forebrain neurons is associated with severe defects in learning and memory. In addition to the behavioral abnormalities described above, mice that lack G9a/GLP in postnatal forebrain become severely obese and almost double their body weight as compared to littermate controls by five to six months of age (Schaefer et al. 2009).

Importantly, the severe behavioral abnormalities caused by G9a/GLP deficiency in the postnatal forebrain in mice mimic most of the key signs of the human 9q34 mental retardation syndrome. In humans, haploinsufficiency in GLP most likely will affect both the development and function of adult neurons. Our findings, however, suggest that deficiency of GLP in postnatal forebrain neurons is sufficient to cause complex behavioral abnormalities in mice (Schaefer et al. 2009). This finding indicates a possible role for GLP regulation of adult brain function, including cognition, motor activity and mood regulation, in normal healthy individuals. It has been demonstrated recently that protein levels and activities of G9a/GLP and the corresponding brain-expressed H3K9me2-specific demethylases can be regulated by oxygen levels (Chen et al. 2006b), metal ions (Fe, NiCl2; Chen et al.

2006a; Ke et al. 2006; Yamane et al. 2006), and the abundance of endogenous metabolites such as alpha-ketoglutarate (Yamane et al. 2006). It is possible that changes in local oxygen or metabolite concentrations in the brain of healthy individuals may affect changes in brain function due to alteration of H3K9me2-controlled gene expression. Therefore, identification of genes directly controlled by G9a/GLP may help to elucidate the genetic mechanisms controlling the affected complex behaviors in mice and man.

## 4 Transcriptional de-Differentiation of Adult Neurons Lacking H3K9me2

The task of precise characterization of gene expression in the brain is greatly complicated by the morphological and functional heterogeneity of neuronal and non-neuronal subpopulations. Recent technological advances have made it possible to characterize mRNA expression patterns in small and functionally distinct cell populations (Doyle et al. 2008; Heiman et al. 2008). This technology, called TRAP (Translating Ribosome Affinity Purification), utilizes cell type-specific tagging of the large ribosomal subunit protein L10 with the enhanced green fluorescent protein (eGFP). Expression of an eGFP-tagged ribosomal protein selectively in neurons of interest is followed by immunoaffinity purification and extraction of the polyribosome bound, cell type-specific mRNAs from crude brain extracts (Doyle et al. 2008; Heiman et al. 2008). Unlike traditional approaches, the TRAP methodology has the advantage of combining the detection of translated mRNAs with cell-type specificity. The methodology also avoids lengthy and frequently inefficient cell separation procedures that, besides severing the mRNA containing axons and dendrites, also promote RNA degradation and cell death. The use of established bacTRAP mouse lines ensures that the mRNA translational profiles can be reproducibly obtained and directly compared from the same neuronal cell population in experimental and control mice.

Using Drd1 and Drd2 neuron-specific ribosomal-associated mRNA profiling (bacTRAP technique), we found that the loss of euchromatic H3K9me2 in adult Drd1 or Drd2 neurons led to relatively modest changes in gene expression (Schaefer et al. 2009). From over 12,000 Drd1 or Drd2 neuron-expressed genes, only ~200-250 displayed expression changes >2 fold. Consistent with the function of H3K9me2 as a suppressor of gene expression in other cells, the majority of changed genes (~80 %) in G9a/GLP-deficient neurons were upregulated upon loss of H3K9me2. Among the genes that show increased expression in the absence of H3K9me2 were a large number (~85 %) that were usually not expressed in neurons. Many of the genes that are ectopically expressed in G9a/GLP-deficient neurons are specific for skeletal and cardiac muscle development and function (Titin, Myosin heavy chain 1 and 6, Troponin 2, Musk), the immune system (Zeta-chain-associated protein kinase 70, B-cell scaffold protein with ankyrin repeats 1, Beta-defensin 1), embryonic liver development (Alpha-fetoprotein, Afamin), and cell growth and proliferation (Brca1,

Brca2; Schaefer et al. 2009). In addition to the transcriptional de-differentiation of Drd1 and Drd2 neurons, lack of G9a/GLP up-regulates the expression of a small fraction (15 %) of neuronal genes, several of which are involved in serotonin and dopamine synthesis and function. Upregulation of several key regulatory enzymes involved in the synthesis of serotonin from tryptophan (Tdo2, Indo1, Ddc) and dopamine from tyrosine (Ddc, Th) could effectively change the balance of these neurotransmitters and their neuro-active metabolites in the brain. The latter feature of G9a/GLP-deficient neurons could contribute at least in part to some of the apathy and depressive-like behavioral alterations, such as anhedonia and impaired reward, reduced locomotor activity and exploration, and impaired social interactions, that are observed in the G9a/GLP mutant mice (Schaefer et al. 2009).

## 5 Conclusions and Discussion

Epigenetic control of brain function is an emerging field of neuroscience. With each neuron being potentially different from another, and with the constant flow of information through the brain, the epigenetic regulators are likely to play a central role in transmitting information about the environment to the chromatin. Our findings point to a key role for G9a/GLP in maintenance of neuronal transcriptional homeostasis and suggest that signals controlling the activity or expression of these enzymes in the adult brain may have a direct and selective impact on regulation of motor activity, cognition and mood.

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# The Role of Histone Acetylation in Long-Term Memory Storage

**Shane G. Poplawski and Ted Abel**

**Abstract** Long-term contextual memory formation requires transcription within the hippocampus, a brain structure deep inside the medial temporal lobe. The mechanisms by which this transcription is initiated and maintained are still poorly understood, although histone acetylation is known to be involved. The interaction between phosphorylated CREB and the histone acetyltransferase CREB-binding protein (CBP) is necessary for long-term memory. Histone acetylation is known to increase at specific CREB regulated promoters after contextual fear conditioning, and histone deacetylase (HDAC) inhibitors, which increase histone acetylation, have been demonstrated to increase long-term memory when injected into the hippocampus during memory consolidation. Specific lysine residues on histone tails are targets for acetylation, and individual acetylation patterns may be important regulators of long-term memory. Determining the genes regulated by histone acetylation after contextual learning will provide insight into the necessary components of memory consolidation and may lead to development of novel, selective therapeutics that enhance memory in diseases in which memory is affected.

## 1 Contextual Learning Induces CREB-Dependent Changes in Gene Expression in the Hippocampus

A role for the hippocampus in forming episodic memories in humans was first described by Scoville and Milner (1957) over 50 years ago. Our understanding of the role of the hippocampus in long-term memory has since been greatly supported by rodent studies. Contextual fear conditioning is a behavior that allows for dissection of particular phases of memory in the hippocampus due to the temporal

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resolution of using only one brief training session (Maren 2001; Maren and Quirk 2004). Contextual fear conditioning consists of placing a mouse in a novel context that is paired with an aversive foot shock. The memory is then tested by measuring freezing, a species-specific fear response, at one hour after training for short-term memory and 24 hours after training for long-term memory. Contextual fear memory formation depends on at least two brain systems: the hippocampus and the amygdala (Maren and Quirk 2004). Hippocampal lesions impair acquisition (Phillips and LeDoux 1992) and produce temporally graded retrograde amnesia (Anagnostaras et al. 1999; Kim and Fanselow 1992) for contextual fear memory. Thus, components of contextual fear memory traces appear to be formed and stored in the hippocampus for weeks prior to systems-level consolidation into the cortex (Frankland and Bontempi 2005).

We and others have focused on identifying the molecular mechanisms in the hippocampus and the amygdala that underlie contextual fear memory storage. In both of these brain regions, there are two time windows after learning with increased phosphorylation of the transcription factor cAMP-response element-binding protein (CREB), which activates this protein and is thought to mediate memory-promoting transcription (Stanciu et al. 2001). These two windows of CREB phosphorylation, 0–30 min and three to six hours after training, coincide strikingly well with the two time windows during which inhibition of transcription or translation impairs memory storage (Bourtchouladze et al. 1998; Igaz et al. 2002). Although CREB is a necessary transcription factor regulating the transcription required for long-term memory formation, studies have shown that CREB phosphorylation alone is not sufficient to drive expression of target genes (Chawla et al. 1998), indicating that additional coactivators of CREB are required for transcription of target genes.

## 2 The Role of Histone Acetyltransferases in Long-Term Memory

Several proteins that interact with phosphorylated CREB are histone acetyltransferases (HATs), including CBP, p300 and p300/CBP associated factor (PCAF). The concept of DNA-histone complexes regulating memory was first proposed in 1975 (Portelli 1975). Histone acetylation appears to play a critical role in a number of psychiatric and neurological disorders, including depression, schizophrenia and intellectual disability (Abel and Zukin 2008; Brennan and Abel 2008). Therefore, these HATs may be crucial regulators of the transcription necessary for long-term memory. Studies have shown that each of these HATs serves a role in distinct types of memory formation. Mice lacking *Pcaf* display short-term memory deficits that gradually worsen with age (Maurice et al. 2008). Although this finding may appear to link PCAF HAT function with memory, short-term memory is transcription-independent and therefore this deficit is likely due to developmental

defects in the knockout animals. Morphological analysis of the mutant mice showed loss of cells in CA1 and CA3 of the hippocampus, further supporting the notion of developmental defects causing memory impairments in these mice (Maurice et al. 2008). Mice overexpressing a truncated form of *p300* (Oliveira et al. 2007) and mice with conditional *p300* deletion (Oliveira et al. 2011) demonstrate selective long-term memory deficits in both contextual fear conditioning, which is hippocampus- and amygdala-dependent, and object recognition memory, which is hippocampus-independent (Oliveira et al. 2010). Hippocampus-dependent spatial memory is not affected in either of these mice, which indicates p300 is not required for this form of memory and may suggest that the memory deficits in the p300 mutant mice are due to transcriptional effects in brain regions outside the hippocampus.

The most thoroughly studied HAT in memory is CREB binding protein (CBP). CBP is a transcriptional coactivator that is recruited to phosphorylated CREB (Kwok et al. 1994) as well as other transcription factors (Parker et al. 1999). Mutations of *Cbp* in the human population cause a form of intellectual disability termed Rubinstein-Taybi syndrome (Petrij et al. 1995). Recent studies by our lab and others have demonstrated that the HAT CBP is required for efficient long-term memory consolidation (Alarcon et al. 2004; Korzus et al. 2004; Oike et al. 1999 Wood et al. 2005, 2006a). Six independently generated *Cbp* alleles disrupt long-term memory without impairing short-term memory (Wood et al. 2005, 2006a; Alarcon et al. 2004; Korzus et al. 2004; Oike et al. 1999; Bourtchouladze et al. 2003; Valor et al. 2011). We have produced mice in which CBP activity in neurons is reduced by the transgenic expression of an inhibitory form of CBP lacking the HAT domain (Wood et al. 2005). These mice exhibit selective deficits in long-term contextual memory, underscoring the importance of the HAT activity of CBP for memory consolidation. As a coactivator with intrinsic HAT activity, CBP interacts with numerous transcription factors and contains multiple functional domains. Importantly, mice homozygous for a mutation in the kinase-inducible interaction (KIX) domain of CBP, in which CBP is unable to interact specifically with the CREB/ATF transcription factor family, are impaired in long-term memory formation (Wood et al. 2006a; Stefanko et al. 2009). Mice with forebrain-specific full cbp deletion appear to display both short- and long-term memory deficits (Chen et al. 2010), a result that has not been observed with any previous *Cbp* mutant mice. However, viral deletion of CBP in adult mice causes deficits in synaptic plasticity and selectively in a long-term form of hippocampus-dependent memory (Barrett et al. 2011). This finding would indicate that the deficits in short-term memory observed in mice with the CaMKIIα-driven forebrain deletion are probably due to developmental defects rather than to a role of CBP in short-term memory. Taken together, the studies of the role of the HAT CBP in long-term memory consolidation suggest that histone acetylation may be a crucial regulator of transcription during memory consolidation.

## 3 Acetylation of Specific Lysine Residues on Histone Tails May Be Important During Memory Consolidation

Because individual HATs are known to acetylate specific lysine residues on histone tails, the possibility exists that acetylation of particular lysine residues controls the transcription necessary for long-term memory. Although the first evidence for histone acetylation occurring during memory consolidation came by studying the incorporation of radioactive acetyl-CoA into histones in 1979 (Schmitt and Matthies 1979), the first study to demonstrate changes in particular histone acetylation marks was made in 2004, with the finding that acetylation of lysine 14 on histone H3 increased in bulk histone extracts one hour after contextual learning (Levenson et al. 2004). It has since been found that acetylation of lysine 12 of histone H2B and lysine 12 of histone H4 are both increased in bulk histone extracts during spatial memory consolidation (Bousiges et al. 2010). The latter mark is particularly interesting, because a study by Peleg et al. (2010) found multiple acetylation marks upregulated one hour after contextual fear conditioning, including lysine 9 of histone H3 and lysines 5, 8 and 12 of histone H4. These changes were studied in both young mice, which are able to properly form long-term memories, and aged (16 months old) mice, which have long-term memory deficits. Lysine 12 of histone H4 was the only mark specifically increased in young mice but not in aged mice, indicating that this acetylation mark may be a key regulator of the transcription necessary for long-term memory formation.

The most direct method to determine the histone acetylation marks pertinent to memory formation would be to study those affected by the histone acetyltransferases that are known to be required during memory consolidation, such as CBP. In vitro, CBP and p300 have multiple overlapping lysine targets on histone tails. However, recent in vivo work has suggested a more circumscribed role for individual HATs in regulating acetylation of specific lysine residues. Work by Jin et al. (2011) has found that knockout of PCAF in mouse embryonic fibroblast cells surprisingly only decreases acetylation lysine 9 of histone H3, whereas loss of CBP/p300 showed remarkable specificity for decreasing acetylation of lysines 18 and 27 of histone H3. It is important to note that changes in acetylation of lysines 18 and 27 of histone H3 were not examined in previous studies of bulk histone acetylation changes after learning. The observed specificity of CBP/p300 histone acetylation in fibroblasts could be due to compensation by other HATs at a subset of CBP/p300 target lysines or the guiding of HAT activity by accessory components that provide target specificity. In either scenario, the targets of specific HATs may be different in the hippocampus, an anatomical site where memory is consolidated. To bypass these problems, Barrett et al. (2011) have used a focal viral deletion of CBP to study the histone marks regulated by CBP in the adult mouse hippocampus. Viral deletion has the benefit of temporal control, which limits compensation by other HATs, as well as stereotaxic control to only affect the brain region of interest. Using this technique, acetylation differences were observed on lysine 14 of histone H3, lysine 12 of histone H2B, and lysine 8 of histone H4 but not the mark that is

decreased in aged mice, lysine 12 of histone H4 (Barrett et al. 2011). Lysines 18 and 27 of histone H3 were not studied in this work. Determining the genes regulated by these acetylation marks during memory consolidation promises to uncover interesting targets that are important for long-term memory, and future methods targeting these specific lysine residues could hold potential for novel therapeutics that would improve memory formation while limiting side effects.

## 4 Histone Deacetylase Inhibitors Increase Long-Term Memory

The increases in histone acetylation during memory consolidation suggest that artificially increasing histone acetylation could enhance long-term memory. Histone acetylation at a promoter is controlled through a delicate balance of HATs, such as CBP, which add acetyl groups to specific lysine residues on histone tails, and histone deacetylases (HDACs), which remove acetyl groups from these lysines (Fig. 1).

Increasing histone acetylation could be achieved either through enhancing HAT activity or by reducing memory-suppressing HDAC activity. HDAC inhibitors, which increase histone acetylation, enhance long-term memory when given during memory consolidation. Our work has shown that the memory enhancement by the HDAC inhibitor trichostatin A (TSA) requires the CREB-CBP interaction, indicating that CREB target genes are those required for the memory enhancement. Surprisingly, TSA increases expression of only two of fourteen CREB target genes examined, the orphan nuclear receptors *Nr4a1* and *Nr4a2* (Vecsey et al. 2007). It was previously shown that knock-down of *Nr4a2* expression impairs long-term memory formation in a spatial discrimination task (Colon-Cesario et al. 2006; McQuown et al. 2011), and constitutive, global, heterozygous *Nr4a2* knockout mice are impaired in long-term memory formation in the hippocampus-dependent passive avoidance task (Rojas et al. 2007), indicating that this gene expression change may be important for the memory-enhancing effects of HDAC inhibitors (Hawk and Abel 2011).

Determining the molecular targets and pathways affected by HDAC inhibitor treatment promises to uncover new genes necessary for contextual memory as well as provide novel avenues for therapeutic intervention for diseases in which memory is affected. Studying the role of particular HDACs in memory formation is another strategy that could lead to development of selective pharmacological agents that cause memory enhancement. HDACs are classified into four families (I-IV) based on sequence homology and structure (Fischer et al. 2010). Current studies indicate a role for class I HDACs in long-term memory formation. Class I HDACs act in the nucleus and include HDACs 1–3 and 8. Genetic evidence has demonstrated that both HDAC2 and HDAC3, but not HDAC1, are required for long-term memory (Guan et al. 2009; McQuown et al. 2011). Our lab has used a pharmacological approach to demonstrate that a Class I-selective HDAC inhibitor, MS275, enhances long-term spatial memory when injected into the hippocampus after training (Hawk et al. 2011). MS275 has highest affinity for HDACs 1 and 2, moderate affinity for

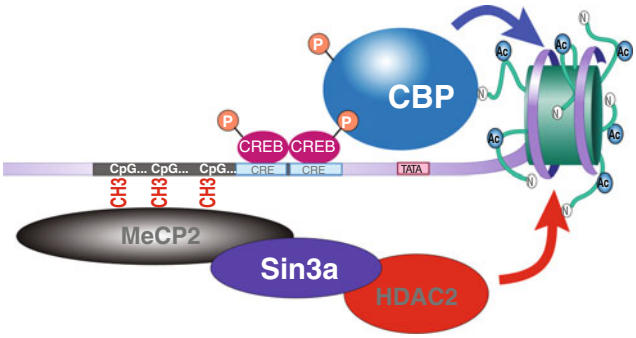


**Fig. 1 Regulation of histone acetylation at promoters of genes necessary for long-term memory.** After learning, CREB is activated by phosphorylation, binds to CREB response elements (CREs) in the genome, and recruits the coactivator CBP to the region. Acetyl groups are added to lysine residues on histone tails by the histone acetyltransferase (HAT) function of CBP. Acetylation is removed by class I histone deacetylase (HDAC) proteins

HDAC3, and low affinity for HDAC8 (Hawk et al. 2011), suggesting that inhibition of HDAC2 alone may enhance memory formation. Another HDAC inhibitor that preferentially binds to HDAC3, RGFP136, has also been shown to enhance long-term memory (McQuown et al. 2011). It is currently unclear whether HDAC2 and HDAC3 have overlapping genetic targets important for memory formation or whether each regulates expression of distinct genes. Targeting only those specific HDAC proteins that dampen memory formation could lead to the application of selective therapeutics with greatly reduced side effect profiles compared to broad-spectrum HDAC inhibitors. This development is especially important considering that chronic treatment of broad-spectrum HDAC inhibitors, such as TSA, causes synaptic dysfunction (Nelson et al. 2006).

## 5 Overview and Future Directions

Histone acetylation is required during memory consolidation, but how this transient epigenetic modification that occurs within 30 minutes of training affects memory 24 hours after training is still a matter of debate. An exciting possibility is that transient changes in histone acetylation after learning prime targets for long-lasting DNA methylation changes. This process could convert a rapid gene expression change into a long-lasting mark responsible for memory maintenance. Numerous studies have demonstrated a link between DNA methylation and histone acetylation (Detich et al. 2003; Dong et al. 2007; Miller et al. 2008). A major mechanism of DNA methylation-mediated repression is the recruitment of methyl-DNA binding proteins, such as MeCP2, to regulatory elements. MeCP2 binds SIN3A, which then recruits HDAC1 and HDAC2 to methylated DNA (Nan et al. 1998). Experiments in dissociated cortical neurons have demonstrated that neuronal depolarization causes

active *Bdnf* promoter DNA demethylation, which is followed by dissociation of MeCP2-SIN3A-HDAC complexes and increased *Bdnf* transcription (Chen et al. 2003; Martinowich et al. 2003). This result suggests an intriguing feed-forward loop whereby histone acetylation could cause DNA demethylation to generate a long-lasting loss of histone deacetylase activity at memory-promoting genes. A link beween histone acetylation and DNA methylation is supported by studies examining DNA methylation changes as a result of maternal behavior (Weaver et al. 2004). Pups from high-grooming mothers display low DNA methylation of the glucocorticoid receptor exon $1_7$ promoter, and pups from low-grooming mothers display high DNA methylation levels. High methylation of the glucocorticoid receptor was associated with increased stress responsiveness. Treatment of pups from low-grooming mothers with the HDAC inhibitor TSA increased histone acetylation at the exon $1_7$ promoter, rescued DNA hypermethylation, and reversed the stress phenotype caused by poor maternal care. Thus, these results demonstrate that a change in histone acetylation at a given promoter can affect DNA methylation at the same promoter. This study also demonstrates that HDAC inhibitors are a viable pharmacological manipulation for rescue of the behavioral consequences of an adverse epigenetic state.

Studying the mechanisms regulating transcription during memory consolidation promises to uncover previously unknown pathways involved in long-term memory and may offer new therapeutic targets for intervention in diseases in which memory is affected. The transcription factor CREB and its coactivator CBP are essential elements in the transcriptional program that supports the formation of hippocampus-dependent memories. CBP is a HAT that can alter the epigenetic landscape in promoters important for memory to increase histone acetylation and drive increased gene expression. Both histone acetylation and DNA methylation have been demonstrated to have important roles in controlling gene expression during memory consolidation, and pharmacological agents exist that can alter both processes. The possibility that epigenetic mechanisms interact to control gene expression during consolidation and to poise genes for later reactivation will be an exciting area of future study.

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# DNA Methylation in Memory Formation

**J. David Sweatt**

**Abstract** The mechanism by which behavioral memories are able to endure in a structure as dynamic as the brain is a long-standing mystery of cognition. For example, a memory must survive, by some mechanism, the ongoing replacement of the proteins that were initially responsible for its formation. In this review I describe the beginning of work designed to test the hypothesis that the brain utilizes DNA methylation as a mechanism to contribute to the stable maintenance of memories. Decades ago, both Francis Crick and Robin Holliday speculated that DNA methylation might be a self-perpetuating mechanism involved in memory storage. Ongoing studies in a number of laboratories are testing the idea that learning-induced epigenetic modifications in the cortex can serve as stable alterations in brain cells, contributing to the support of memory stability. Thinking in analogy to developmental biology, oncogenesis, and cellular differentiation, investigators in this area have begun to pursue the possibility that chromatin- and DNA-modifying molecular mechanisms might play a role in memory in the adult CNS.

## 1 Introduction

One important goal in contemporary studies of cognition is to understand the molecular mechanisms that contribute to long-term memory consolidation and storage. An emerging idea is that the regulation of chromatin structure, mechanistically via histone modifications, may contribute to long-lasting behavioral change (Levenson et al. 2004; Alarcon et al. 2004; Korzus et al. 2004; Wood et al. 2006). We find this idea fascinating because similar mechanisms are used for triggering and storing long-term "memory" at the cellular level, for example, when cells

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differentiate. In the area of memory formation specifically, recent studies have found a role for histone post-translational modifications in regulating long-term synaptic plasticity and long-term memory formation (Jiang et al. 2008; Sweatt 2009; Graff and Mansuy 2008; Barrett and Wood 2008). More recently, we and others have begun to investigate whether another chromatin-regulating mechanism, DNA methylation, is also a dynamic process involved in controlling long-lasting changes in synaptic function and behavior.

A variety of preliminary results motivate this hypothesis at this point (Miller and Sweatt 2007; Miller et al. 2008; Lubin et al. 2008). For example, DNA methyltransferase inhibitors (DNMTi) applied acutely into the adult CNS alter methylation of the *BDNF*, *PP1*, and *reelin* genes at specific loci in upstream CpG islands and block long-term potentiation (LTP) induction in the hippocampal slice preparation. Investigators also have observed that direct hippocampal infusion of DNMTi blocks hippocampus-dependent long-term memory formation in vivo. Moreover, several laboratories have obtained biochemical evidence that long-term memory consolidation is associated with altered DNA methylation in the hippocampus (Miller and Sweatt 2007; Miller et al. 2008; Lubin and Sweatt 2007; Lubin et al. 2008). These results, along with recent results from Meaney's group among others (Meaney and Szyf 2005a, b; Weaver et al. 2005; Nelson et al. 2008; Levenson et al. 2006), suggest the need for a shift in our thinking about DNA methylation. As opposed to being a static mechanism set up during development as a permanent change, DNA methylation appears to be subject to ongoing regulation in the adult nervous system, subject to control by environmental influences, and playing a role in the acute formation of memory. The idea that chemical modification of DNA is an integral component of the molecular processes underlying long-term behavior change, and a description of recent results that test important predictions of this concept, will be the focus of this review. The studies to be described include approaches at the molecular, cellular, electrophysiological, systems neuroscience, and behavioral levels.

## 2 DNA Methylation as a Candidate Stable Molecular Mark in Cells

DNA methylation and histone modifications are the two most extensively investigated epigenetic mechanisms (Fig. 1). Until recently, it was thought that, once laid down, these epigenetic marks remained unchanged for the lifetime of the organism, although recent studies have challenged this view (as will be discussed in this review). Nevertheless, it is clear that DNA methylation and attendant changes in chromatin structure are capable of self-regeneration and self-perpetuation, necessary characteristics for a stable molecular mark. These processes are accomplished in part by the action of DNA methyltranferases (DNMTs). DNMTs can recognize a hemi-methylated C-G dinucleotide (i.e., methylated on only one strand of the DNA) and can convert the complementary C-G on the opposite strand into a methylated C-G. Through this mechanism, DNA can be perpetually methylated in

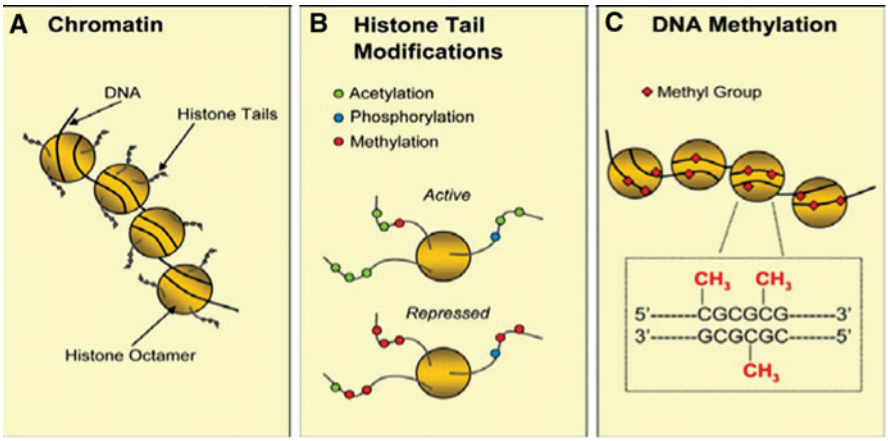


**Fig. 1** Schematic representation of epigenetic marks. A, DNA is condensed within the nucleus through interactions with histones. The DNA–protein complex is referred to as chromatin. B, The N-terminal tail of a histones have several sites for epigenetic marking that can promote or repress gene transcription. C, Methylation of DNA in which a methyl group (red diamonds) is transferred to cytosines in genomic regions in and around gene promoters rich in cytosine-guanine nucleotides (CpG islands). From Jiang et al. 2008

the face of ongoing turnover of molecular marks, in a fashion very analogous to the self-perpetuating auto-phosphorylation of activated CaMKII that had been previously identified as a candidate molecular mechanism for memory storage in the CNS (Roberson and Sweatt 1999).

For this reason, many years ago Francis Crick proposed that a self-perpetuating biochemical autoconversion of methylated DNA might serve as a memory mechanism at the molecular level (Crick 1984). Indeed, this idea was contemporaneous with John Lisman's hypothesis for self-perpetuation of CaMKII autophosphorylation, and the two theories complemented each other. Crick's idea has lain largely unaddressed since then, with the exception of one further paper discussing the theoretical concept published by Robin Holliday, one of the founding fathers of the epigenetics field, and a more recent treatment by Tollefsbol (Holliday 1999; Liu et al 2009). These prior discussions lay the foundation for recent motivation to test the idea of DNA methylation having a role in memory formation and stabilization. DNA methylation is one of the few biochemical mechanisms known to exist that can perpetuate itself indefinitely in the face of ongoing molecular turnover. Contemporary memory researchers in several instances are intrigued by the idea that this mechanism might play a role in memory stabilization.

Regulated DNA methylation occurs in regions of the genome rich in CpG dinucleotides (i.e., CpG islands) that are often found in the promoter region of genes. The family of enzymes that carry out DNA methylation, the DNMTs, comes in two variants: maintenance DNMTs, including DNMT1, and de novo DNMTs, including DNMT3a and 3b. The function of de novo DNMTs is to methylate previously unmethylated CpG sites, whereas the maintenance DNMTs methylate hemi-methylated DNA. DNA methylation is most often associated with

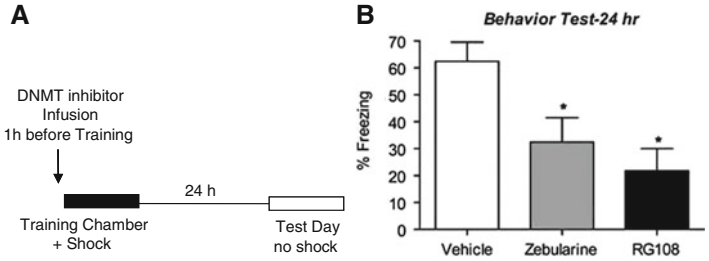


**Fig. 2** DNMT inhibition blocks long-term memory formation. A, DNMT inhibitors (zebularine or RG108) were infused bilaterally into the dorsal hippocampus one hour before contextual fear conditioning training. Vehicle- or drug-treated animals were trained by paired presentation of a novel context with footshock, and freezing behavior was assessed 24 hours later as an index of the animals' long-term memory for the training event. B, Both zebularine and RG108 significantly attenuated ($p < 0.05$) long-term associative fear conditioning 24 hours later, as indicated by diminished freezing behavior. In additional experiments (not shown), post-training infusion of yet another DNMT inhibitor (5-aza-2-deoxycytidine) also attenuated long-term memory formation in the same paradigm

transcriptional silencing, but there are also instances in which DNA methylation can have an activating role (Chahrour et al. 2008). Regardless of the specific scenario, DNA methylation has proven to be a key regulator of gene transcription.

## 3 Recent Evidence Implicating DNA Methylation in Memory Consolidation

Several pieces of evidence are now available that support the idea that DNA methylation plays a role in memory function in the adult CNS. Work by Levenson et al. (2006) demonstrated that general inhibitors of DNMT activity alter DNA methylation in the adult brain and alter the DNA methylation status of the plasticity-promoting genes *reelin* and *bdnf*. Additional studies demonstrated that de novo DNMT expression was upregulated in the adult rat hippocampus after contextual fear conditioning and that blocking DNMT activity blocked contextual fear conditioning (Miller and Sweatt 2007; Lubin et al. 2008; see Fig. 2). In addition, fear conditioning is associated with rapid methylation and transcriptional silencing of the memory suppressor gene *Protein Phosphatase 1* (*PP1*) and demethylation and transcriptional activation of the plasticity genes *bdnf* and *reelin*. These findings have the surprising implication that both active DNA methylation and active demethylation might be involved in long-term memory consolidation in the adult CNS.

More recent work has also begun to investigate the mechanistic interplay between histone acetylation and DNA methylation (Miller et al. 2008). In this study, inhibition of DNMT activity blocked histone acetylation, along with memory consolidation, as previously reported (Levenson et al. 2006; Miller and Sweatt 2007). These deficits, however, were rescued by pharmacologically increasing histone acetylation prior to DNMT inhibition. Finally, a recent series of studies found that the *BDNF* gene locus is also subject to memory-associated changes in DNA methylation and that this effect

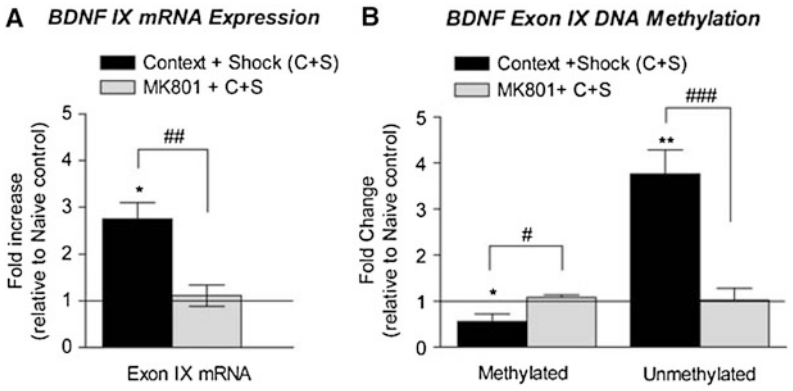


**Fig. 3** Alterations in BDNF gene transcription and DNA methylation with contextual fear conditioning. A, BDNF gene expression (total exon IX mRNA) increases with contextual fear conditioning (Context plus Shock, C + S, black bars). The increased transcription is NMDA receptor-dependent, as indicated by a blockade by MK801 (grey bars). B, BDNF exon IX DNA methylation was assessed using methylation-specific primers for either the methylated (left two bars) or unmethylated (right two bars) state of the gene. Both primer sets revealed that fear conditioning is associated with exon IX demethylation and that the demethylation is blocked by NMDA receptor blockade. Asterisks (*) indicate different from naïve control ($p < 0.05$), cross-hatches (#) indicate MK801 different from trained animals ($p < 0.05$, 0.01, or 0.005)

is regulated by the NMDA receptor (Lubin et al. 2008; see Fig. 3). Together, these findings lend strong support to the idea that DNMT activity is not only necessary for memory but that DNA methylation and histone modifications work together to regulate memory formation in the adult CNS.

Overall, these various results suggest that DNA methylation is dynamically regulated in the adult CNS in response to experience and that this cellular mechanism is a crucial step in memory formation. It is important to note that these findings suggest that memory formation involves both increased methylation at memory suppressor genes and decreased methylation at memory promoting genes. Thus, memory function might be driven by either hypermethylation or hypomethylation. Overall, these observations suggest that DNMT activity is necessary for memory and that DNA methylation may work in concert with histone modifications to regulate memory formation and storage in the adult CNS, in particular in the hippocampus and neocortex, which are known sites of memory formation and storage.

## 4 Regulation of DNA Demethylation

The idea of the occurrence of active DNA demethylation has been contentious. Traditional epigenetic mechanisms and studies have posited only passive DNA demethylation as a result of cell division and failure to replicate DNA methylation marks. Active demethylation through direct chemical removal of methyl groups on cytosines (or methylcytosines themselves) has been proposed by several groups,

including those of Szyf and Meaney (Meaney and Szyf 2005a, b; Weaver et al. 2005; Nelson et al. 2008; Levenson et al. 2006), but this concept has been controversial (Ooi and Bestor 2008).

However, several pieces of recent information motivate investigating a potential role for active DNA demethylation in non-dividing cells in the mature CNS. First, we have obtained indirect evidence of active DNA demethylation in the adult CNS in response to behavioral training (fear conditioning) using non-quantitative methods, such as PCR-based methods and methylation-dependent immunoprecipitation (Miller and Sweatt 2007; Lubin et al. 2008). This evidence has led us to hypothesize the existence of rapid, active DNA demethylation in the adult CNS. Second, two recent publications (Kangaspeska et al. 2008; Métivier et al. 2008) have demonstrated rapid DNA demethylation and re-methylation, referred to as "cycling" of methyl-cytosine (MeC) in cultured cells. This demethylation occurs too rapidly to be explained by passive demethylation through cell division and must therefore be due to an active demethylation process. These investigators have also proposed a specific demethylation mechanism: C-to-T conversion of MeC, followed by base-excision repair of the resulting nucleotide mismatch. Most recently, exciting work from Song and colleagues supported this idea (Ma et al. 2009a, b); these investigators have demonstrated that DNA repair mechanisms are utilized to demethylate DNA in non-dividing neurons, specifically through base-excision repair mechanisms controlled by the Growth Arrest and DNA Damage 45 (GADD45-beta) regulatory system. This finding demonstrates that demethylation can occur independently of DNA replication and in a terminally differentiated neuron. Moreover, two new studies (Kriaucionis and Heintz 2009; Tahiliani et al. 2009) have shown that a novel DNA base, hydroxymethyl-cytosine, may uniquely occur in the CNS and may serve as a precursor nucleoside for active demethylation. Thus, there is now a substantial body of evidence supporting the idea that active DNA demethylation can occur in non-dividing neurons these findings make viable the idea that active control of DNA methylation may play a role in activity-dependent processes in the CNS.

## 5 How Does Altered Methylation Lead to Altered Neuronal Function? The LTP Hypothesis

The studies described above implicate DNA methylation as a regulator of memory formation in vivo, but what is the cellular basis for this effect? Available data suggest that one component contributing to DNMT-inhibitor-induced memory deficits is disruption of hippocampal LTP (Levenson et al. 2006). For example, in an early series of studies it was observed that Zebularine and 5-aza-2-deoxycytidine, inhibitors of DNMT activity, blocked the induction of LTP at Schaeffer collateral synapses in the hippocampus (Levenson et al. 2006). The ability of DNMT inhibitors to affect synaptic function has since been confirmed and extended by Lisa Monteggia's group (Nelson et al. 2008). Overall these findings are also consistent with a variety of additional findings demonstrating

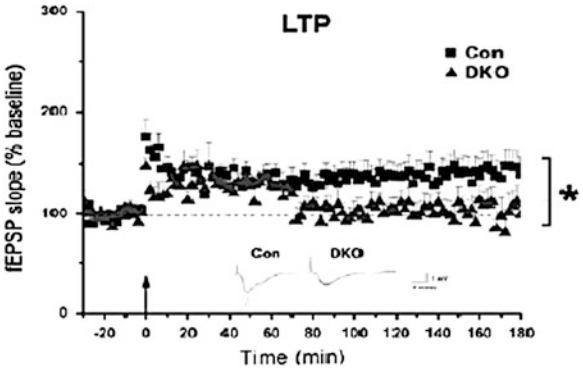


**Fig. 4** Impaired synaptic plasticity in DNMT1/3a double KO mice. LTP at Schaffer/collateral synapses was reduced in adult DKO mice (* $P < 0.05$). Field EPSP (fEPSP) slopes in control (squares) versus DKO mice (triangles) were recorded 30 min before and 180 min after tetanic stimulation (100 Hz, 1 sec). Scale bar, 5 msec/1 mV. In additional studies (not shown), we observed normal basal synaptic transmission in *Dnmt* mutant mice and normal long-term plasticity in single *Dnmt1* KO mouse brain. Input–output curves from the DKO, *Dnmt1* SKO as well as wildtype control groups were essentially identical, as shown by plotting the synaptic responses against presynaptic fiber volley amplitudes

that manipulation of the methyl-DNA binding protein MeCP2 leads to disruption of long-term synaptic plasticity in the CNS (Collins et al. 2004; Moretti et al. 2006).

Thus, these prior results have demonstrated that application of a variety of DNMT inhibitors leads to deficits in hippocampal LTP and deficits in memory (Levenson et al. 2006; Miller and Sweatt 2007). The principal limitation to these pharmacological studies of LTP (and also the studies described above regarding hippocampus-dependent long-term contextual learning) is the concern that DNMT inhibitors might be having non-specific cytotoxic effects or off-target effects. A recent series of studies has tested whether conditional knockout of the DNMT1 and DNMT3a genes (in combination) leads to deficits in LTP at Schaffer collateral synapses in area CA1. For these studies, floxed-allele DNMT1 and DNMT3a mice were crossed with a CaMKII promoter-driven cre line to knock out DNMTs post-natally, selectively in forebrain neurons. Hippocampal slices prepared from animals deficient in DNMT1/DNMT3a exhibited deficits in LTP as assessed at Schaffer/collateral synapses using tetanic stimulation (Feng et al 2010; see Fig. 4). Taken together, these various findings provide a potential cellular locus for the effects of DNMT inhibition on memory formation, that is, that disruption of LTP and other forms of hippocampal synaptic plasticity may underlie the memory-blocking effects of DNMT inhibitors.

Moreover, behavioral assessments of these conditional knockout animals indicated deficits in contextual fear conditioning and the Morris water maze (Feng et al. 2010). These data strongly suggest that the behavioral memory effects seen using pharmacologic inhibition of DNMTs are a result of DNMT inhibition as opposed to off-target effects. Overall these data are in good agreement with the

broad hypothesis that DNA methylation plays a role in regulating synaptic plasticity and memory formation.

However, these findings do not provide an explanation for the effects of DNMT inhibition at the neural systems and circuit level. One current hypothesis is that DNA methylation controls the stability of hippocampal place fields as one mechanism whereby the effects on synaptic plasticity may be manifest as a disruption of hippocampal circuit function. Testing this hypothesis in the future will yield important insight into a potential higher-order role for DNA methylation, that is, stabilizing neural circuit connectivity in the CNS.

As described in this section, available data suggest that regulation of hippocampal DNA methylation is critical for memory formation. However, consistent with the limited temporal role of the hippocampus in maintaining memory, prior studies have found that altered methylation in hippocampus returns to basal levels within one day of learning (Miller and Sweatt 2007). Therefore, recent studies have focused on a brain region thought to support the storage of long-lasting memories to begin to test the specific hypothesis that DNA methylation might contribute to long-term memory maintenance.

## 6 Implications for Memory Storage at the Cellular Level

The advent of sophisticated molecular, genetic and cellular techniques has lent itself to a relatively deep understanding of how memories are initially formed. In stark contrast, however, is our limited understanding of how these same memories are maintained by the brain. A variety of recent studies have used contextual fear conditioning, a form of hippocampal associative learning, to explore the hypothesis that changes in the cortex support long-lasting memories (Frankland et al. 2004; Restivo et al. 2009; Wang et al. 2009). These recent studies have indicated that contextual fear conditioning in rodents results in a memory that persists for many months, during which time the associative memory transitions from "recent" to "remote." This change is thought to represent system consolidation, in which the memory shifts from the hippocampus to a long-term dependence on the dorsomedial prefrontal cortex (dmPFC). The dmPFC is subdivided into two main sub-components: the anterior cingulate cortex (ACC) and the prelimbic cortex.

In pioneering work in a 2004 study, Frankland et al. investigated what effect inactivating the ACC would have on fear memory retrieval at various post-training time points. Interestingly, intra-ACC infusions at both 18 and 36 days (remote memory), but not at one or three days post-training (recent memory), interfered with retrieval. This finding suggests that system consolidation of contextual fear memory occurs between 3 and 18 days and further supports the system consolidation view of contextual fear memory. These findings also imply that a tangible persisting molecular marker must exist in the dmPFC that subserves the preservation of remote memory.

To begin characterizing the role of DNA methylation in the dmPFC (ACC and prelimbic cortex) following hippocampus-dependent learning, in a recent study we

examined this brain region for memory-associated changes in the pattern of methylation in the promoter of memory-associated genes with large, CG-rich CpG islands (Miller et al. 2010). These studies began to directly test whether remote memory consolidation in the cortex is associated with altered DNA methylation at gene loci known to be important for memory. We examined the pattern of methylation in the promoter of three memory-associated genes (*zif268, reelin* and *calcineurin*; Fig. 5a-c) at one hour, one day and seven days after training animals for contextual fear conditioning. Importantly, in these preliminary studies no retrieval test was given before sacrifice, except in a subgroup of animals tested to confirm the presence of fear memory at seven days.

In these studies, we observed that the immediate early gene *zif268*'s promoter was demethylated in all groups (context only, shock only and context plus shock-trained animals) at all time points (Fig. 5d). Thus, a variety of environmental stimuli (exploration of a novel environment, mild footshock and associative training) are capable of producing a lasting change in the methylation state of *zif268* in the dmPFC. In addition, *zif268* transcript was elevated above naïve control levels in all animals (Fig. 5g) except those sacrificed without a retrieval test seven days after training. This result is consistent with the gene's pattern of demethylation at both time points, the dmPFC's response to novelty and learning, and the rapid reflection of cellular activation provided by immediate early genes (IEGs). The promoter region of *reelin*, a positive regulator of memory (Weeber et al. 2002; Beffert et al. 2005), was hypermethylated in trained animals within an hour of training and remained methylated for at least seven days, though levels shifted toward controls at the later time points (Fig. 5e). In concordance with the increase in transcription-repressing cytosine methylation, levels of *reelin* transcript were reduced in trained animals one hour after training and one hour after a seven-day post-training retrieval test (Fig. 5h). Methylation of the phosphatase and memory suppressor (Malleret et al. 2001), *calcineurin*, was unchanged in the prefrontal cortex an hour after training. However, the gene's promoter became hypermethylated within one day of training and continued to increase over the next seven days (Fig. 5f), though transcript levels were equivalent to controls (Fig. 5i).

To determine if the lasting cortical methylation is truly a reflection of associative learning, we gave animals pre-training injections of the NMDA receptor antagonist MK-801. A subgroup of animals tested seven days post-training for contextual fear memory confirmed the ability of NMDA receptor antagonism to interfere with acquisition of a fear memory (data not shown). MK-801 also prevented the seven-day *reelin* and *calcineurin* hypermethylation, without affecting *zif268* (data not shown), providing further support that the *reelin* and *calcineurin* hypermethylation is in specific response to associative environmental signals. These data are in agreement with the hypothesis that gene-specific DNA methylation occurs in response to associative learning and further demonstrate that this process is engaged in multiple brain regions.

In their 2004 study, Frankland et al. investigated what effect inactivating the ACC would have on fear memory retrieval at various post-training time points. Interestingly, intra-ACC infusions at both 18 and 36 days (remote memory), but not

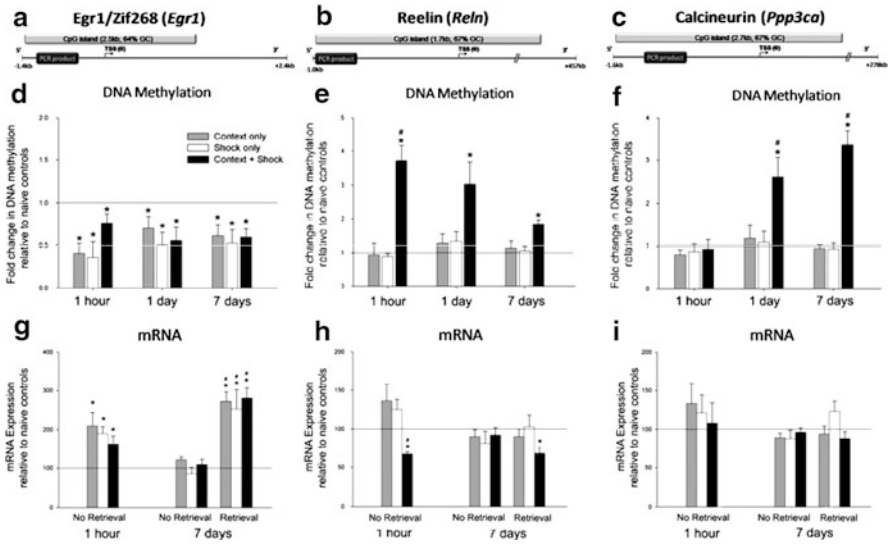


**Fig. 5** DNA methylation and gene transcription changes in the dmPFC following training for contextual fear conditioning. a-c, Location of promoter CpG islands analyzed in mDIP assay for *Egr1/Zif268* (*a*), *reelin* (*b*) and *calcineurin* (*c*). d, Relative to naïve controls, all treatment groups showed demethylation of *Egr1/zif268* at all time points (1 h: $F_{(3,\ 18)} = 22.99$, $P < 0.001$; 1 day: $F_{(3,\ 19)} = 14.99$, $P < 0.001$; and seven days post-training: $F_{(3,\ 25)} = 15.50$, $P < 0.001$). * post hoc comparisons, $P < 0.001$. e, Relative to all treatment groups, *reelin's* promoter is hypermethylated in trained animals at all time points (1 h: $F_{(3,\ 18)} = 29.05$, $P < 0.001$; one day: $F_{(3,\ 19)} = 6.63$, $P < 0.005$; and seven days post-training: $F_{(3,\ 25)} = 10.58$, $P < 0.001$). * post hoc comparisons, $P < 0.05$. seven days post-training, *reelin's* hypermethylation is significantly less than at one hour ($F_{(2,\ 20)} = 6.36$, $P < 0.01$; #). f, Relative to all treatment groups, *calcineurin's* promoter is hypermethylated in trained animals 24 hours and seven days post-training (1 h: $F_{(3,\ 18)} = 0.27$, $P > 0.05$; one day: $F_{(3,\ 19)} = 5.73$, $P < 0.01$; and seven days post-training: $F_{(3,\ 25)} = 33.52$, $P < 0.001$). * post hoc comparisons, $P < 0.05$. One and seven days post-training, *calcineurin's* hypermethylation is significantly greater than at one hour ($F_{(2,\ 20)} = 13.96$, $P < 0.01$; #). g, *Egr1/zif268* transcript is elevated in all treatment groups one hour after training (relative to naïve controls—context: $t_4 = 3.11$, shock: $t_4 = 5.10$, context + shock: $t_4 = 3.02$; * $P < 0.05$) and following a retrieval test seven days after training (relative to naïve controls—context: $t_4 = 6.95$, shock: $t_4 = 3.12$, context + shock: $t_6 = 6.59$; * $P < 0.05$) (relative to seven-day no retrieval groups—$F_{(5,\ 27)} = 11.97$, # post hoc comparisons $P < 0.005$). h, Relative to all treatment groups, *reelin* transcript is significantly lower in trained animals one hour after training (relative to naïve controls—$t_4 = -9.53$, * $P < 0.005$; relative to context and shock controls—$F_{(2,\ 12)} = 6.14$, # $P < 0.05$). Following a retrieval test seven days after training, trained animals' *reelin* transcript was significantly lower than naïve controls ($t_6 = -4.00$, * $P < 0.01$). i, *Calcineurin* transcript is equivalent across all groups. N's = 4–8 animals per group

one or three days post-training (recent memory), interfered with retrieval. This finding suggests that system consolidation of contextual fear memory occurs between 3 and 18 days and also that the cortical DNA methylation events we observe during the first week post-training are appropriately timed to participate in the initial laying down of a memory trace in the cortex, perhaps tagging specific neurons to be used for subsequent downloading of memories from the

hippocampus. Thus, to further characterize the source of learning-induced cortical methylation, we infused the NMDA receptor antagonist APV directly into the hippocampus (CA1) immediately before training (Sanders and Fanselow 2003). APV not only interfered with learning but also blocked the *reelin* and *calcineurin* methylation present in the dmPFC seven days after training (data not shown), indicating that a single hippocampus-dependent learning experience is sufficient to drive lasting, gene-specific DNA hypermethylation in the cortex. These findings are in agreement with the system consolidation view of contextual fear memory and present a tangible marker (DNA methylation) that can be used for a learning-induced dialogue between the hippocampus and dmPFC.

To support memory persistence, DNA methylation in the cortex would need to be long-lasting. Therefore, in an additional experiment we examined the persistence of a fear memory and the methylation status of these genes 30 days after training. Increased methylation of *calcineurin's* promoter in trained animals was observed at this later time point, as was a preservation of decreased *zif268* methylation. *Reelin*, however, exhibited a declining pattern of methylation, such that it was equal to controls by 30 days. These results indicate that a single hippocampus-dependent learning event marks genes in the prefrontal cortex for a minimum of 30 days, well into the time period that memories are thought to become dependent upon this region. We also examined transcript levels of these three genes following a one-month post-training retrieval test. Memory retrieval-associated *Calcineurin* mRNA was specifically reduced in trained animals whereas *zif268* transcription increased in all groups. These observations are in good agreement with the alterations in DNA methylation we observed at these gene sites.

Finally, as an additional test of whether DNA methylation in the dmPFC is necessary for the maintenance of a remote memory, we examined the effect on memory of inhibiting DNMTs 30 days after training. Beginning on post-training day 29, animals received an intra-ACC infusion of the DNMT inhibitor (DNMTi) 5-azadeoxycytidine (5-aza) every 12 hours for a total of three infusions (Fig. 6a). DNMTi animals failed to display normal fear memory in the retrieval test given one hour after the final infusion (Fig. 6b), supporting the idea that methylation in the ACC is critical to the support of remote memory.

Overall, these results suggest the intriguing hypothesis that methylation helps to maintain memories and may indeed serve as a marker of the memory trace.

## 7 Summary and Open Questions

The overarching hypothesis described in this review is that DNA methylation controls the stability of plastic changes at the circuit and behavioral levels. In this vein, recent studies using molecular approaches have investigated whether transient and/or persisting changes in DNA methylation occur in the mature CNS in response to learning experiences. In addition, various studies have used pharmacology or gene engineering to test whether methylation is necessary for stable changes in circuit function and behavioral memory.

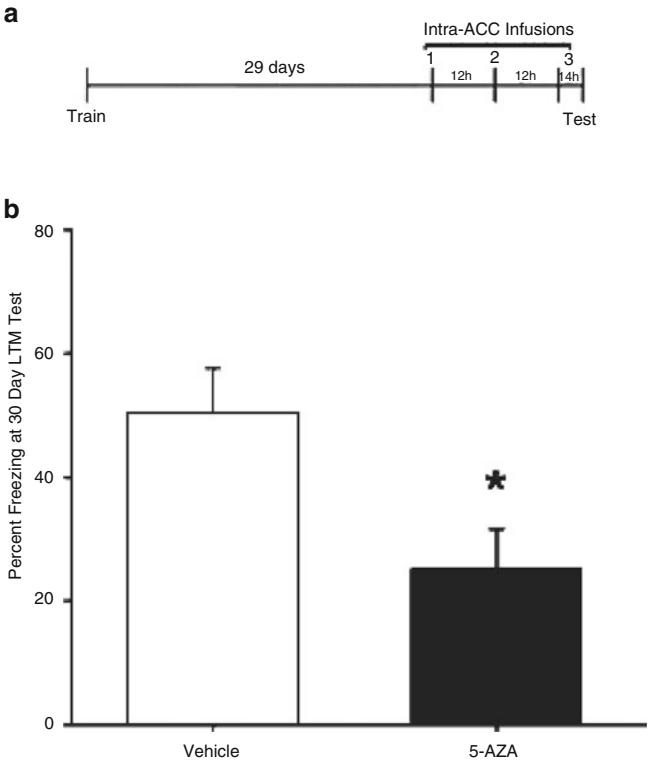


**Fig. 6** Inhibiting DNMTs interferes with the anterior cingulate's ability to support remote memory. a, Schematic representation of the experimental design. b, Intra-ACC infusions of 5-aza 30 days after training disrupted remote fear memory (vehicle N = 14, 5-aza N = 14; $F_{(1,27)} = 7.01$). * $P < 0.05$

Based on these studies we and others propose that DNA chemical modification is plastic in non-dividing, terminally differentiated neurons in the adult CNS. If these ideas are true, a redefinition of several of the fundamental tenets of epigenetics will be required, introducing the idea of plasticity of DNA methylation and the idea that epigenetics-related molecular mechanisms are functionally active in terminally differentiated, non-dividing cells. It is worth noting that, thus far, these ideas have met with significant resistance from the epigenetics community.

An additional consideration for the neuroscience community is that a central organizing concept of physiological psychology, at least regarding behavioral change, is the principle of Hebbian synaptic plasticity as the central mechanism underlying behavioral modification. Recent work on the role of epigenetic, nucleus-based changes being required for memory may necessitate re-thinking certain aspects of this fundamental hypothesis. A single adult neuron can have 10,000

synapses but has only one nucleus. Within the nucleus there are only one or two copies of a given specific gene. Chemical modification of DNA implies a state change for the entire neuron. Chemically modifying and silencing or activating a given gene can alter the genomic complement of the entire cell. If chemical modification of genes underlies behavioral plasticity, this implies a re-thinking of the fundamental assumption that synaptic plasticity is the only locus driving behavioral modification. At a minimum, there may be two levels of mechanism in play: one residing at the individual synapse and one residing at the epigenome and operating cell-wide.

Studies implicating dynamic regulation of DNA methylation in mature, non-dividing cells in the mature nervous system has also helped prompt new investigations into the biochemical mechanisms of DNA demethylation. Identifying the biochemical and biophysical mechanisms underlying DNA demethylation is a compelling topic of great relevance. However, addressing these mechanisms will require detailed studies of the organic chemistry and enzymology of nucleoside covalent modification and various hypothetical mechanisms are under consideration at this point (Day and Sweatt 2010).

To date studies in this area have not attempted to determine, in a comprehensive fashion, how DNA methylation at the cellular level gets translated into altered circuit and behavioral function. Most studies thus far have used a candidate target gene approach to identify specific sites of methylation changes. However, these data only allow the assessment of a small subset of changes in DNA methylation in the CNS. At present it is not fruitful to try to mechanistically tie these specific changes at single gene exons to complex multicellular, multicomponent processes like LTP, place field stabilization, and behavioral memory, because of the limitation that the molecular approaches are sampling such a small subset of genes. A full understanding of how altered DNA methylation gets translated into functional molecular changes in the cell will require further investigation in the future, involving whole-epigenome molecular approaches.

However, the studies executed thus far do give mechanistic insights. For example, the available data indicate that DNA methylation in a specific brain region, the ACC, controls remote memory stability. These studies thereby serve as a foundation for formulating future specific hypotheses concerning how DNA methylation might control persisting changes in synaptic and neural circuit function at the molecular level and ultimately for understanding how transient or persisting changes in DNA methylation manifest themselves in altered neuronal function. Finally, the direct demonstration of a persisting molecular mark in the cortex is not a trivial finding in its own right. Few, if any, persisting changes in molecular marks have ever been demonstrated in the cortex in memory formation. Even persisting cortical synaptic potentiation has never been directly demonstrated in association with memory. For this reason, we feel that the direct demonstration of persisting changes in DNA methylation in the cerebral cortex provides an important proof-of-principle observation concerning the hypothesis that persistent covalent chemical modifications might underlie a subset of persisting molecular changes in remote memory.

However, these studies have not addressed several additional, important questions concerning how DNA methylation might be contributing to memory storage. The mechanisms by which DNA methylation might control memory storage are not clear. For instance, is methylation altering a neuron's basal state, thus altering its response to future stimuli? An example of this might be the lowering of a neuron's firing threshold, which transcriptional repression of *calcineurin* may accomplish, given its importance in long-term depression and interference with long-term potentiation (Malleret et al. 2001). An additional possibility is that synaptic proteins and signaling pathways downstream of neuronal activation utilize methylation as a mechanism to self-perpetuate through the regulation of their own transcription rate. For example, methylation may support the perpetuation of glutamate receptor exchange at synapses tagged by potentiation (McCormack et al. 2006; Kessels and Malinow 2009). These possibilities need not be mutually exclusive. Regardless of the specific answers to these questions, studies executed thus far are but a small set of first steps toward demonstrating that DNA methylation is a self-perpetuating signal utilized by the brain to preserve remote memories, a finding that has important implications for the question of how memories are maintained.

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# Epigenetic Mechanisms Regulating Memory Formation in Health and Disease

**Li-Huei Tsai**

**Abstract** In 1984, Francis Crick (1916–2004) proposed that "memory might be coded in alterations to particular stretches of chromosomal DNA" (Crick Nature 312:101, 1984). Although the response to this idea was relatively modest at the time, 20 years later it was shown that histone acetylation, a common form of epigenetic modification, was dynamically altered during memory formation (Levenson et al. J Biol Chem 279:40545–40559, 2004). Histone acetylation is regulated by the opposing activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs; Bird Nature 447:396–398, 2007; Berger et al. Genes Dev 23:781–783, 2009). HAT proteins are subdivided into five families that have high sequence similarity and related substrate specificity (Kimura et al. J Biochem 138:647–662, 2005). HDACs belong to an ancient protein family that is found in archea, eubacteria, plants, fungi and animals and that requires a $Zn^{2+}$ ion as a cofactor. Based on phylogenetic analysis, HDACs are grouped into three classes (Class I, II and IV).

## 1 Histone Deacetylases and Cognition

Class I HDACs, in particular HDAC1, 2 and 3, are primarily found within the nucleus, where they regulate histone acetylation and suppress gene expression. These HDACs are recruited to the promoter/enhancer regions of genes via transcriptional repressor and co-repressor proteins (de Ruijter et al. 2003). Transient increases in the occurrence of specific histone modifications have been suggested to

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influence distinct mechanisms, such as initiation of transcription or transcriptional elongation (Peleg et al. 2010). Recent work implicates histone acetylation as having an important role during learning and memory processes. Rodents display a transient increase in histone acetylation after exposure to various learning paradigms (Levenson et al. 2004; Chwang et al. 2006; Fischer et al. 2007; Vecsey et al. 2007; Fontan-Lozano et al. 2008; Koshibu et al. 2009; Peleg et al. 2010), and synaptic plasticity and memory formation are facilitated in wildtype mice and rats after treatment with HDAC inhibitors, suggesting that increased histone acetylation facilitates cognitive function(Levenson et al. 2004; Fischer et al. 2007; Vecsey et al. 2007; Peleg et al. 2010).

Recently, it was shown that the upregulation of H4K12 acetylation induced by behavioral training is reduced in aged mice. The acute administration of SAHA to these mice was able to restore both learning-induced H4K12 acetylation and memory function, whereas the HDAC1-selective inhibitor, MS-275, failed to do so (Peleg et al. 2010). These data suggest that H4K12 acetylation is an early biomarker for an impaired genome-environment interaction in the aging brain.

The overexpression of HDAC2 in mouse neurons results in striking impairments in memory formation and synaptic plasticity, which are not observed in HDAC1-overexpressing mice (Guan et al. 2009). These mice also exhibit reduced hippocampal H4K12 and H4K5 acetylation, whereas other marks, such as AcH3K14, are not affected. HDAC2 knockout (KO) mice, but not HDAC1 KO mice, likewise exhibit increased H4K12 and H4K5 acetylation, enhanced learning and memory, and synaptic plasticity and serve as a rare model of cognitive enhancement. Thus, HDAC2 seems to play a major role in learning and memory and synaptic plasticity, as well as in the regulation of H4K12, whose dysregulation is causatively implicated in age-associated memory impairment (Peleg et al. 2010).

## 2 p25/Cdk5 And Neurodegeneration

The proline-directed serine/threonine kinase, Cdk5, has been well characterized as a tau kinase (Baumann 1993; Paudel et al. 1993) that associates with neurofibrillary tangles in the brain of Alzheimer's disease (AD) patients (Pei et al. 1998; Augustinack et al. 2002). Cdk5 is activated when bound to one of its two activators, p35 or p39, which have related primary sequences (Tsai et al. 1994; Tang et al. 1995; Nikolic et al. 1996; Chae et al. 1997; Kwon and Tsai 1998; Ko et al. 2001; Wenzel et al. 2007). Nearly a decade ago, it was revealed that the truncated form of p35, p25, was increased in the brains of AD patients (Patrick et al. 1999; Tseng et al. 2002). Subsequently, the increased generation of p25 was found in mouse models of AD (Otth et al. 2002; Saura et al. 2004; Kitazawa et al. 2005; Oakley et al. 2006; Zhang and Simpkins 2010a, b), ischemia (Lee et al. 2000), amyotrophic lateral sclerosis (Nguyen et al. 2001), Parkinson's disease (Smith et al. 2003), Huntington's disease (Paoletti et al. 2008), and hippocampal sclerosis (Sen et al. 2006; Muyllaert et al. 2008). In cultured mouse neurons, the cleavage of p35 to p25 is induced upon exposure to a number of neurotoxic insults, including hydrogen

peroxide ($H_2O_2$; oxidative stress), glutamate (excitotoxicity), ionomycin (high internal calcium), and Aβ (amyloid toxicity; Lee et al. 2000). Therefore, p25 generation is associated with neurotoxicity both in vitro and in vivo.

## 3 CK-p25 Mouse Model

In 2003, we generated a bi-transgenic mouse model that expressed a p25-GFP fusion protein in an inducible, post-developmental, and forebrain-specific manner (CK-p25 mouse; Cruz et al. 2003). Upon the induction of p25 expression, neurodegenerative events occur in a rapid and predictable manner. Increased BACE1 activity and Aβ generation are evident by two weeks of induction, astrogliosis is observed after four weeks of induction, neuronal loss and cognitive impairment are appreciable after six weeks of p25 induction, and severe tau pathology is evident after prolonged (>20 weeks) p25 expression (Cruz et al. 2003; Fischer et al. 2005). In addition, following p25 induction, neurons aberrantly express cell cycle proteins and form double-strand DNA breaks at an early stage prior to their death. The onset of p25 overexpression is followed by the eventual death of nearly 40 % of hippocampal and cortical neurons in this mouse model.

## 4 HDAC Inhibitor Treatment Reinstated Learning and Restored Remote Memory After Neurodegeneration

Treatment of the CK-p25 mice with the non-selective HDAC inhibitor sodium butyrate significantly improves cognitive performance in mice even after severe neurodegeneration has occurred (Fischer et al. 2007). The HDAC inhibitors SAHA and phenylbutyrate (PB) have also been shown to reinstate learning behavior in a mouse model of AD (APPswe/PS1dE9 mice; Kilgore et al. 2009; Ricobaraza et al. 2009). This treatment, which resulted in the recovery of learning ability, did not affect amyloid pathology itself. Rather, mice treated with PB displayed elevated H4 acetylation, accompanied by an increased production of proteins implicated in synaptic function (Ricobaraza et al. 2009). Treatment with various HDAC inhibitors has thus emerged as a promising new strategy for therapeutic intervention in neurodegenerative disease (Selvi et al. 2010; Chuang et al. 2009; Mai et al. 2009).

To investigate the target of the small molecule HDAC inhibitors in enhancing cognition, we used a combination of pharmacological and mouse genetic approaches and identified that a class I HDAC, HDAC2, but not HDAC1, plays an important role in regulating synaptic plasticity and memory formation (Guan et al. 2009). Chromatin immunoprecipitation experiments revealed that HDAC2 associates with the promoter of many genes implicated in synapse formation/remodeling and memory formation and likely suppresses their expression (Guan et al. 2009; Fig. 1). These results suggest that HDAC inhibitors enhance cognitive function by inhibiting HDAC2 to render HDAC2 target genes more accessible to

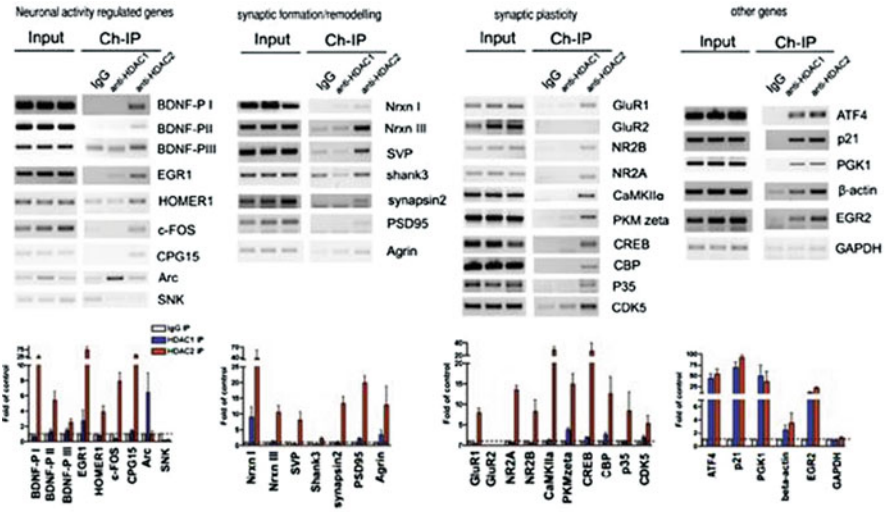


**Fig. 1** Semi-quantitative PCR from chromatin immunoprecipitation (ChIP) of samples showed specificity of DNA binding for HDAC2 and HDAC1 quantified according to the real-time PCR signal. Note that HDAC2 preferentially associates with the promoter of genes regulated by neuronal activity, which is involved in synapse formation and synaptic plasticity

transcription activators and co-activators recruited by neuronal activity stimulation. The data presented at the IPSEN 2011 meeting delineate a novel and important role for HDAC2 activity in the cognitive impairments associated with neurodegenerative disease.

## 5 Conclusion

Our findings suggest a model for an epigenetic mechanism underlying memory impairment in neurodegeneration and provide hope for the beneficial effects of HDAC inhibition. In this model (Fig. 2), neurotoxic insults such as p25, Aβ, and oxidative stress upregulate HDAC2 occupancy on learning and memory genes. Increased HDAC2 occupancy reduces histone acetylation and silences the expression of these genes,leading to cognitive impairment and synaptic loss. The recovery of histone acetylation, however, via HDAC inhibition can restore histone acetylation and the expression of learning and memory and synaptic plasticity-related genes. The restoration of this gene expression leads to a reversal of memory deficits.

These data are exciting because they provide hope for the treatment of neurodegenerative diseases such as AD even when the disease is relatively advanced. A number of recent clinical trials targeting the APP processing pathway in AD have produced disappointing results (Patton et al. 2006; Holmes et al. 2008;

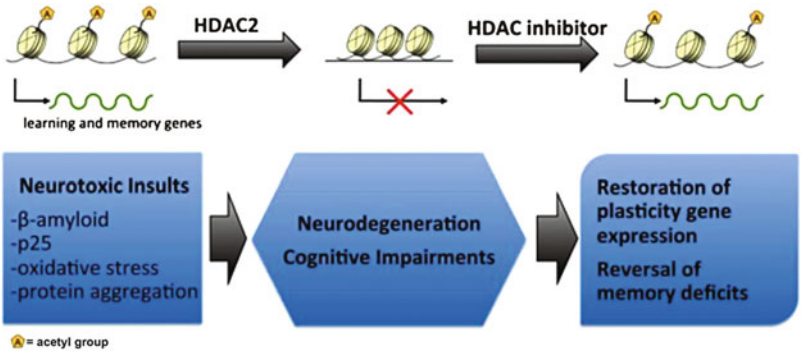


**Fig. 2** A model depicting an epigenetic mechanism underlying memory impairment in neurodegeneration, in which HDAC2 activity leads to the compression of chromatin and the silencing of genes important for learning and memory

Gauthier et al. 2009; Saumier et al. 2009; Talan 2009). One reason given for this setback has been that the patients chosen for the trial were too advanced in their disease and that these therapies would be most beneficial in very early-onset AD. However, an estimated 35 million people worldwide are currently living with dementia, of which AD is the most common cause (Hardy 2006, Alzheimer Disease International 2009, 2010). Should we focus our efforts entirely on preventative or early-onset therapeutics and leave these people to their fate? We believe that a focus upon treatments aimed at ameliorating cognitive symptoms and memory loss, even after neuronal loss has taken place, is equally important in improving the treatment options for people with neurodegenerative disease. In this realm, therapeutics targeting chromatin remodeling enzymes such as the HDACs hold great promise.

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# Genetics and Epigenetics of Autism Spectrum Disorders

**Thomas Bourgeron**

**Abstract** Autism spectrum disorders (ASD) are characterized by impairments in reciprocal social communication and stereotyped behaviors. Genetic and epigenetic studies have identified a relatively large number of genes and biological processes that constitute a comprehensive framework to better understand this heterogeneous syndrome. Based on the most robust findings, three observations can be made. First, genetic and epigenetic contributions to ASD are highly heterogeneous with most likely, at the genetic level, a combination of alleles with low and high penetrance. Second, the majority of the genetic mutations apparently affect a single allele, suggesting a key role for gene dosage in the susceptibility to ASD. Finally, the broad expression and function of the causative genes suggest that alteration of synaptic homeostasis could be a common biological process associated with ASD. The large spectrum of clinical heterogeneity might be due to the inter-individual difference in the capacity of "buffering" deleterious synaptic mutations. A better understanding of the interplay between genetic/epigenetic variations and synaptic homeostasis should shed new light on the causes of ASD and may provide means to modulate the severity of the symptoms.

## 1 Introduction

The diagnosis of autism is based on impairments in reciprocal social communication and stereotyped behaviors. The term "autism spectrum disorders" (ASD) is used to refer to any patient whose illness meets these diagnostic criteria. But beyond

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this unifying definition lies an extreme degree of clinical heterogeneity, ranging from profound to moderate impairments. Indeed, autism is not a single entity, but rather a complex phenotype thought to be caused by different types of defects in common pathways, producing similar behavioral phenotypes. The prevalence of ASD overall is about 1/100, but closer to 1/300 for typical autism (Fernell and Gillberg 2010). ASD are more common in males than females, with a 4:1 ratio (Freitag 2007; Abrahams and Geschwind 2008).

The first twin and family studies performed in the last quarter of the 20th century conclusively described ASD as the most "genetic" of neuropsychiatric disorders, with concordance rates of 82-92 % in monozygotic (MZ) twins versus 1-10 % in dizygotic (DZ) twins; sibling recurrence risk is 6 % (Freitag 2007; Abrahams and Geschwind 2008). However, recent studies have indicated that the concordance for ASD in DZ twins might be higher (>20 %) than previously reported (Hallmayer et al. 2011). Furthermore the concordance for ASD in MZ could also be lower than originally suggested (Lichtenstein et al. 2010; Ronald et al. 2011). All these studies pointed at a larger role for the environment and/or epigenetic factors in the susceptibility to ASD. For example, in a twin study using structured diagnostic assessments (Autism Diagnostic Interview–Revised and Autism Diagnostic Observation Schedule), a large proportion of the variance in liability was explained by shared environmental factors (55 %; 95 % confidence interval (CI), 9 %-81 % for autism and 58 %; 95 % CI, 30 %-80 % for ASD in addition to moderate genetic heritability (37 %; 95 % CI, 8 %-84 % for autism and 38 %; 95 % CI, 14 %-67 % for ASD). However, most likely due to the high genetic and clinical heterogeneity of autism/ASD, the very large CI of the results leaves the gene/environment debate unresolved.

From a cognitive perspective, 15-70 % of children diagnosed with ASD have intellectual disabilities (ID) (Gillberg and Coleman 2000), and it is now understood that autism symptoms can be caused either by gene mutations or by chromosomal aberrations. Indeed, in approximately 10-25 % of the affected individuals, autism is "syndromic," i.e., occurring in a child with a known genetic or environmental toxin disorder, such as fragile X, tuberous sclerosis, neurofibromatosis, valproate syndrome, or autism caused by brain herpes simplex infection (Freitag 2007; Gillberg and Coleman 2000). An interesting proposition made by David Skuse (2007) is that the apparent association between intellectual disability and autistic syndromes is not because they usually have common causes but rather because the presence of both features greatly increases the probability of clinical ascertainment. In the last few years, various independent studies and large-scale international efforts have identified a growing number of candidate genes for ASD and suggested a set of mechanisms that could underlie the ASD phenotype. In this chapter, I will briefly review the recent advance in understanding the genetic and epigenetic susceptibility to ASD. Finally, I will discuss recent results supporting the hypothesis that an alteration in synaptic homeostasis could be one of the mechanisms leading to ASD.

## 2 Genetics and ASD

Due to the absence of classical Mendelian inheritance, ASD were first thought to be a polygenic trait involving many genes, each one with low effect. Therefore, model free linkage studies, such as affected sib-pair analyses, were performed to identify susceptibility genes. Many genomic regions were detected, but only a restricted number of loci were replicated in independent scans (e.g., chromosome 7q31 and 17q11; Bourgeron 2009; Toro et al. 2010). To homogenize the genetic and phenotypic data and to gain higher statistical power, collaborative efforts were initiated. By using these approaches, several genes were associated with ASD. A list of 219 genes is available at AutDB, a public, curated, web-based database for autism research (http://www.mindspec.org/autdb.html). However, most of these genes remain only candidates since their association has not always been confirmed by replication and/or functional validation. Depending on the impact of the mutation on the risk for ASD (a property called penetrance), two main categories of genes can be defined. In the first category, genes or loci appear to have a high penetrance but are mutated in a limited number of individuals (sometime a single individual). In this category, variations are mostly composed of de novo or rare point mutations, copy number variations (CNVs) and cytogenetically detected deletions/duplications (Table 1). The second category of genes includes the so-called susceptibility genes to ASD (Table 2). Here, the variations are mostly composed of single nucleotide polymorphisms (SNPs) or inherited CNVs observed in the general population and associated with low risk for ASD (Fig. 1). In this category of genes especially, the association with ASD should be taken with great care, since the three largest genome-wide association studies (GWAS) performed on more than 1,000 patients in each study could not detect the same genes associated with ASD (Wang et al. 2009; Weiss et al. 2009; Anney et al. 2010).

## 3 Epigenetics and ASD

Epigenetics is defined as a change in gene expression caused by mechanisms other than modification in the underlying DNA sequence. Examples of such changes might be DNA methylation or histone deacetylation or methylation, both of which serve to regulate gene expression without altering the sequence of the genes (Fig. 1). These epigenetic hallmarks may remain through cell divisions and might also pass through the germ cells and last for multiple generations. Among the epigenetic processes, genomic imprinting is a genetic phenomenon by which certain genes are expressed in a parent of origin-specific manner. Several lines of evidence suggest that abnormal epigenetic regulation could contribute to the susceptibility to ASD.

The influence of epigenetics factors in ASD was first suggested by the absence of 100 % concordance for ASD in MZ twin pairs. Derived from a single fertilized egg, the MZ twins are considered to be genetically identical. Thus, discordance in MZ

**Table 1** Genes associated with high risk for ASD

| Gene | Chromosome | Function | Evidence | Inheritance | Diagnosis | References |
|---|---|---|---|---|---|---|
| *FMR1* | Xq27 | Synaptic translation | Mutations | De novo (permutations) | ASD, Fragile X syndrome | Garber et al. 2008 |
| *MECP2* | Xq26 | Chromatin remodeling | CNV, mutations | De novo (rarely inherited) | ASD, Rett syndrome | Amir et al. 1999 |
| *TSC1* | 9q34.13 | mTOR / PI3K pathway | CNV, mutations | De novo, inherited | ASD, Tuberous sclerosis | Wiznitzer 2004 |
| *TSC2* | 16p13.3 | mTOR / PI3K pathway | CNV, mutations | De novo, inherited | ASD, Tuberous sclerosis | Wiznitzer 2004 |
| *NF1* | 17q11.2 | mTOR / PI3K pathway | CNV, mutation | De novo, inherited | ASD, Neurofibromatosis | Rosser and Packer 2003 |
| *PTEN* | 10q23.31 | mTOR / PI3K pathway | CNV, mutations | De novo, inherited | ASD, Cowden syndrome | Butler et al. 2005 |
| *CACNA1C* | 12p13.33 | Calcium channel | Mutation | De novo | ASD, Timothy syndrome | Splawski et al. 2006 |
| *DPYD* | 1p21.3 | Pyrimidine base biosynthesis | CNV | De novo | ASD | Marshall et al. 2008 |
| *RFWD2* | 1q25.1-q25.2 | Ubiquitination | CNV | De novo, inherited | ASD | Glessner et al. 2009 |
| *NRXN1* | 2p16.3 | Synaptic CAM | CNV, mutations, SNP | De novo, inherited | ASD, SCZ | Szatmari et al. 2007; Feng et al. 2006 |
| *CNTN4* | 3p26.3 | Synaptic CAM | CNV | Inherited | ASD, MR | Glessner et al. 2009; Roohi et al. 2009; Morrow et al. 2008; Fernandez et al. 2008 |
| *MEF2C* | 5q14.3 | Transcription factor | CNV, mutations | De novo | MR, Seizures | Le Meur et al. 2010 |
| *SYNGAP1* | 6p21.3 | Synaptic Ras GAP | CNV | De novo | ASD, MR | Piton et al. 2008 |
| *CNTNAP2* | 7q35-7q36.1 | Synaptic CAM | CNV, rare variants * | Inherited | ASD, MR, SCZ, TS | Zweier et al. 2009; Alarcon et al. 2008; Arking et al. 2008; Bakkaloglu et al. 2008; Verkerk et al. 2003; Friedman et al. 2008 |
| *DPP6* | 7q36.2 | Dipeptidyl-peptidase activity | CNV | De novo, inherited | ASD | Marshall et al. 2008 |

| | | | | | | |
|---|---|---|---|---|---|---|
| *DLGAP2* | 8p23.3 | Synaptic scaffold | CNV | De novo | ASD | Marshall et al. 2008 |
| *ASTN2* | 9q33.1 | Neuron-glia interaction | CNV | Inherited | ASD, SCZ, ADHD | Glessner et al. 2009 |
| *SHANK2* | 11q13 | Synaptic scaffold | CNV | De novo | ASD | Pinto et al. 2010 |
| *NBEA* | 13q13.2 | Synaptic protein | Translocation | De novo | ASD | Castermans et al. 2003 |
| *UBE3A* | 15q11-q13 | Ubiquitination | CNV | De novo, inherited | ASD | Glessner et al. 2009 |
| *SHANK3 (del 22q13)* | 22q13 | Synaptic scaffold | CNV, mutations | De novo, inherited | ASD, MR, SCZ | Durand et al. 2007; Moessner et al. 2007 Gauthier et al. 2009 |
| *NLGN3* | Xq13.1 | Synaptic CAM | Mutation | Inherited | ASD | Jamain et al. 2003 |
| *IL1RAPL1* | Xp21.3-p21.2 | Synaptic receptor | CNV, mutations | De novo, inherited | ASD, MR | Piton et al. 2008 |
| *NLGN4* | Xp22 | Synaptic CAM | CNV, mutations | De novo, inherited | ASD, MR, TS | Jamain et al. 2008 |
| *PTCHD1* | Xp22.11 | Hedgehog receptor activity | CNV | Inherited | ASD | Marshall et al. 2008 |
| *GRIA3* | Xp25 | Synaptic receptor | CNV | Inherited | ASD | Jacquemont et al. 2006 |

ASD, autism spectrum disorder ; SCZ schizophrenia; MR, mental retardation; ADHD, attention-deficit hyperactivity disorder ; MDC1D, congenital muscular dystrophy; BP, bipolar disorder; TS, Tourette syndrome; * in contrast to mutations, the functional role of the rare variants was not confirmed.

**Table 2** Proposed susceptibility genes for ASD

| Gene | Chromosome | Function | Evidence | Diagnosis | References |
|---|---|---|---|---|---|
| *ASMT* | PAR1 | Melatonin pathway | Inherited CNV, SNPs, mutations | ASD | Melke et al. 2008; Cai et al. 2008; Toma et al. 2007 |
| *DISC1/ DISC2* | 1q42.2 | Axonal growth | Inherited CNV | ASD, SCZ | Williams et al. 2009 |
| *TSNAX* | 1q42.2 | Cell differentiation | Inherited CNV | ASD, SCZ | Williams et al. 2009 |
| *DPP10* | 2q14.1 | Dipeptidyl-peptidase activity | Inherited CNV | ASD | Marshall et al. 2008 |
| *CNTN3* | 3p12.3 | Synaptic CAM | Inherited CNV | ASD | Morrow et al. 2008 |
| *FBXO40* | 3q13.3 | Unknown function | Inherited CNV $P = 3.3 \times 10^{-3}$ | ASD | Glessner et al. 2009 |
| *SLC9A9* | 3q24 | Transporter | Inherited CNV, mutations | ASD, ADHD, MR | Morrow et al. 2008 |
| *PCDH10* | 4q28 | Synaptic CAM | Inherited CNV | ASD | Morrow et al. 2008 |
| *PARK2* | 6q26 | Ubiquitination | Inherited CNV $P = 3.3 \times 10^{-3}$ | ASD, PD | Glessner et al. 2009 |
| *IMMP2L* | 7q31.1 | Mitochondrial protease | Inherited CNV | ASD, TS, ADHD | Petek et al. 2007 |
| *PCDH9* | 13q21 | Synaptic CAM | Inherited CNV | ASD | Marshall et al. 2008; Morrow et al. 2008 |
| *MDGA2* | 14q21.3 | GPI anchor protein | Inherited CNV $P = 1.3 \times 10^{-4}$ | ASD | Bucan et al. 2009 |
| *BZRAP1* | 17q22 | Benzodiazepine receptor binding | Inherited CNV $P = 2.3 \times 10^{-5}$ | ASD | Bucan et al. 2009 |
| *PLD5* | 1q43 | Phospholipase D | SNP rs2196826 $P = 1.1 \times 10^{-8}$ | ASD | Anney et al. 2010 |
| *SLC25A12* | 2q31.1 | Synaptic receptor | SNP rs2056202 $P = 1 \times 10^{-3}$ | ASD | Turunen et al. 2008; Ramoz et al. 2004 |
| *CDH9/ CDH10* | 5p14.2 | Synaptic CAM | SNP rs4307059 $P = 3.4 \times 10^{-8}$ | ASD | Wang et al. 2009 |
| *SEMA5A* | 5p15.2 | Axonal guidance | SNP rs10513025 $P = 2 \times 10^{-7}$ | ASD | Weiss et al. 2009 |
| *TAS2R1* | 5p15.2 | Receptor | SNP rs10513025 $P = 2 \times 10^{-7}$ | ASD | Weiss et al. 2009 |
| *GRIK2* | 6q16.3 | Synaptic receptor | SNP rs3213607 $P = 0.02$ | ASD, SCZ, OCD, MR | Jamain et al. 2002 |
| *POU6F2* | 7p14.1 | Transcription factor | SNP rs10258862 $P = 4.4 \times 10^{-7}$ | ASD | Anney et al. 2010 |
| *RELN* | 7q22.1 | Axonal guidance | GGC repeat in the 5' UTR $P < 0.05$ | ASD, BP | Skaar et al. 2005 |

| | | | | | |
|---|---|---|---|---|---|
| *NRCAM* | 7q31.1 | Synaptic receptor | SNP rs2300045 $P = 0.017$ | ASD | Bonora et al. 2005 |
| *MET* | 7q31.2 | Tyrosine kinase | SNP rs1858830 $P = 2 \times 10^{-3}$ | ASD | Campbell et al. 2006 |
| *EN2* | 7q36.3 | Transcription factor | SNP rs1861972 $P = 9 \times 10^{-3}$ | ASD | Benayed et al. 2005 |
| *ST8SIA2* | 15q26.1 | N-glycan processing | SNP rs3784730 $P = 4 \times 10^{-7}$ | ASD | Anney et al. 2010 |
| *GRIN2A* | 16p13.2 | Synaptic receptor | SNP rs1014531 $P = 2.9 \times 10^{-7}$ | ASD, SCZ | Barnby et al. 2005 |
| *ABAT* | 16p13.2 | Enzyme | SNP rs1731017 $P = 1 \times 10^{-3}$ | ASD, GABA-AT Deficiency | Barnby et al. 2005 |
| *SLC6A4* | 17q11.2 | Serotonin transporter | Meta analysis $P > 0.05$ | ASD, OCD | Devlin et al. 2005 |
| *ITGB3* | 17q21.3 | Cell-matrix adhesion | SNP Leu33Pro $P = 8.2 \times 10^{-4}$ | ASD | Weiss et al. 2006 |
| *TLE2 / TL6* | 19p13 | Wnt receptor signaling pathway | SNP rs4806893 $P = 7.8 \times 10^{-5}$ | ASD, FHM2, AHC | Kilpinen et al. 2009 |
| *MACROD2* | 20p12 | Unknown function | SNP rs4141463 $P = 2 \times 10^{-8}$ | ASD | Anney et al. 2010 |

ASD, autism spectrum disorder; SCZ, schizophrenia; PD, Parkinson's disease; TS, Tourette syndrome; ADHD, attention-deficit hyperactivity disorder; MR, mental retardation; FHM2, familial hemiplegic migraine 2 ; AHC, alternating hemiplegia of childhood; OCD, obsessive-compulsive Ddsorder ; BP, bipolar disorder.

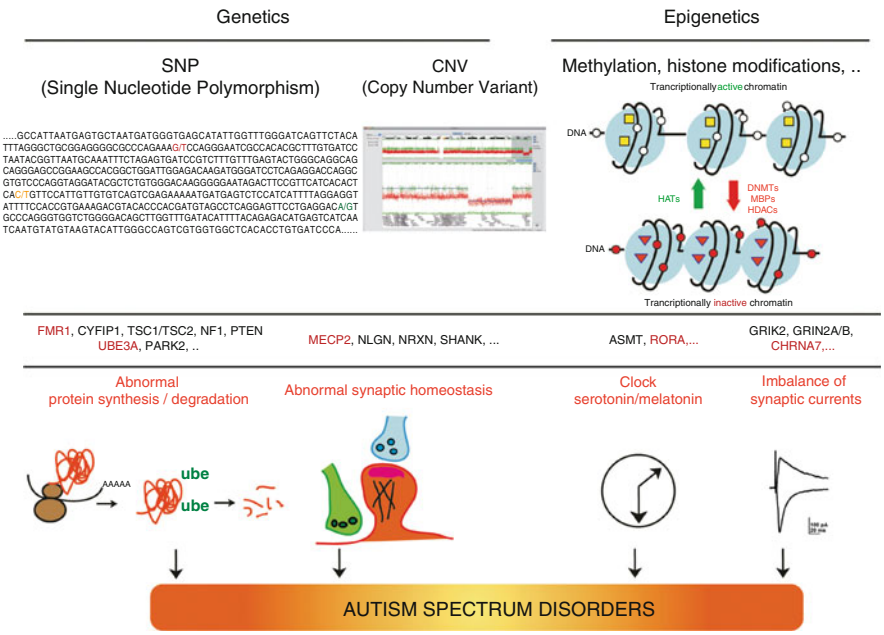


**Fig. 1 Genetic and epigenetic variations associated with ASD and their consequences on brain functions**. Top panel: Genetic variations include single nucleotide polymorphisms (SNP) or copy number variations (CNV). Epigenetic variations include DNA methylation and histone modifications. DNA hypomethylation and histone acetylation are usually associated with open chromatin and active transcription. DNA hypermethylation and histone methylation are associated with heterochromatin and inactive transcription. Middle panel: Example of genes associated with ASD. Genes altered and/or involved in epigenetic regulations are indicated in orange. Bottom panel: The biological pathways associated with ASD (Database list see at the end of the chapter)

twins is generally interpreted as the result of epigenetic and/or environmental factors. However, the phenotypic disparity could result from genetic differences since de novo somatic mutations affecting only the affected MZ twin could occur at early or later stages of his/her development. Indeed, different CNVs were found in MZ twin pairs from the general population, indicating that CNV events could occur during mitosis at an early stage and could therefore contribute to phenotypic differences (Bruder et al. 2008). Interestingly, detection of such CNVs in phenotypically discordant MZ twins might be useful to identify new susceptibility genes for ASD.

If these relatively rare genetic differences among MZ twins do not contribute to the discordance, then a difference in epigenetic regulation might at least in part influence the phenotypic outcome. A recent large-scale methylation profiling revealed several candidate genes differentially methylated between discordant MZ twins as well as between both twins and unaffected siblings (Nguyen et al. 2010). The differentially methylated genes demonstrated enrichment for gene transcription, nervous system development, cell death/ survival,

and other biological processes previously associated with ASD (Nguyen et al. 2010). The difference in methylation levels for two of these genes, BCL-2 and retinoic acid-related orphan receptor alpha (RORA), was further validated by bisulfite sequencing and methylation-specific PCR. In addition, immunohistochemical analyses of tissue arrays containing slices of the cerebellum and frontal cortex revealed a decreased expression of RORA and BCL-2 proteins in patients with ASD compared with age- and sex-matched controls. Interestingly, RORA is involved in the regulation of circadian genes, and abnormalities in clock or in the melatonin pathway have been previously associated with susceptibility to ASD (Bourgeron 2007; Melke et al. 2008). The same authors analyzed the transcriptome and the miRNA of three pairs of MZ twins discordant for a diagnosis of autism: a normal sibling for two of the twin pairs, two pairs of autistic and unaffected siblings, and a pair of unnafected MZ twins (Hu et al. 2006; Sarachana et al. 2010). Several differentially expressed genes (e.g., *CHL1* or *ROBO1*) were compelling candidates for ASD or located in chromosomal regions previously identified by genetic analyses as harboring autism susceptibility genes. In addition, a quantitative relationship was seen between severity of symptoms and expression of several autism candidate genes when twins with classic autism or with milder autistic traits were compared with their respective normal siblings (Hu et al. 2006). Differentially expressed miRNAs were found to target genes highly involved in neurological functions and disorders in addition to genes involved in gastrointestinal diseases, circadian rhythm signaling, and steroid hormone metabolism and receptor signaling (Sarachana et al. 2010). Network analyses of the putative target genes further revealed an association with ASD and other co-morbid disorders, including muscle and gastrointestinal diseases, as well as with biological functions implicated in ASD, such as memory and synaptic plasticity. Putative gene targets (ID3 and PLK2) of two brain-specific miRNAs (hsa-miR-29b and hsa-miR-219-5p) were validated by miRNA overexpression or knockdown assays, respectively. Comparisons of these mRNA and miRNA expression levels between discordant twins and between case–control sib pairs showed an inverse relationship, further suggesting that ID3 and PLK2 are in vivo targets of the respective miRNA. Interestingly, two of the most significantly down-regulated miRNAs (miR-219 and miR-132) have been reported to be involved in modulating the master circadian clock located in the suprachiasmatic nucleus. Brain-specific miR-219 has a role in NMDA receptor signaling (Kocerha et al. 2009) and is a target for the master circadian regulator CLOCK and BMAL1 (Brain and muscle ARNT-like 1) complex. It exhibits robust circadian rhythm expression, and is fine-tuned the length of the circadian period in mice (Cheng et al. 2007).

The second piece of evidence of a role for epigenetic factors in ASD relies on genetic mutations affecting genes involved in epigenetic regulations. The best example is MeCP2 (Fig. 2B), a protein that directly and/or indirectly regulates neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), by binding to methylated DNA (Chahrour et al. 2008). Deletions or mutations of *MECP2* are associated with Rett syndrome in females, whereas duplications of *MeCP2* are

associated with mental retardation and ASD in males and with psychiatric symptoms, including generalized anxiety, depression, and compulsions, in females (Ramocki et al. 2009).

Third, several chromosomal regions associated with ASD are subject to epigenetic regulation, such as parental imprinting. The chromosome 15q11-q13 contains an imprinted region associated with Angelman syndrome, Prader-Willi syndrome and other neuropsychiatric disorders, including ASD (Marshall et al. 2008; Miller et al. 2009; Depienne et al. 2009; van der Zwaag et al. 2010; Shen et al. 2010; Sharp et al. 2008; Masurel-Paulet et al. 2010; Doornbos et al. 2009; Helbig et al. 2009; Szafranski et al. 2010; Ben-Shachar et al. 2009; Stefansson et al. 2008; International Schizophrenia Consortium 2008; Tam et al. 2010; Murthy et al. 2007; Shinawi et al. 2009; van Bon et al. 2009; Pagnamenta et al. 2009; Kirov et al. 2009; de Kovel et al. 2010). The 15q11-q13 region is characterized by recurrent deletions/duplications with breakpoints generally located within five segmental duplications named BP1 to BP5, which act as hotspots of non-allelic homologous recombination. Maternally transmitted duplication of the chromosome 15q is one of the main genetic causes of ASD. Smaller deletions between BP4 and BP5, encompassing the *CHRNA7* locus, have also been associated with a broad range of disorders such as ID, epilepsy, schizophrenia, and ASD (Miller et al. 2009; Shen et al. 2010; Sharp et al. 2008; Masurel-Paulet et al. 2010; Helbig et al. 2009; Ben-Shachar et al. 2009; Stefansson et al. 2008; International Schizophrenia Consortium 2008; Shinawi et al. 2009; van Bon et al. 2009; Pagnamenta et al. 2009). Two independent models of chromosome 15 duplications were engineered (Nakatani et al. 2009; Meguro-Horike et al. 2011). The first is a mouse model carrying a 6.3-Mb-wide interstitial duplication in chromosome 7c that is highly syntenic to human 15q11-13. The mice carrying a paternal duplication of that region displayed poor social interaction, behavioral inflexibility, abnormal ultrasonic vocalizations, and anxiety (Nakatani et al. 2009). In the duplicated segment, a non-coding small nucleolar RNA, MBII52, altered the editing ratio of serotonin (5-HT) 2c receptor pre-mRNA and, as a consequence, intracellular concentration of calcium in responses to 5-HT2cR signaling was significantly increased. For the second model, microcell-mediated chromosome transfer was used to generate a maternal 15q duplication in a human neuronal cell line (Meguro-Horike et al. 2011). Remarkably, in contrast to the gene copy number, the transcript levels of *NDN, SNRPN, GABRB3* and *CHRNA7* were reduced compared with expected levels despite having no detectable alteration in promoter DNA methylation. Therefore, while extra copies of genes are expected to result in increased transcript levels, this model recapitulates the direction of transcriptional alterations previously observed in a human brain sample with maternal 15q duplication (Hogart et al. 2009).

Finally, one of the most studied environmental causes of ASD is prenatal exposure to the commonly used antiepileptic drug valproic acid (VPA; Dufour-Rainfray et al. 2011). Its mechanism of action is related to an increase in brain concentrations of gamma-aminobutyric acid (GABA), the major inhibitory

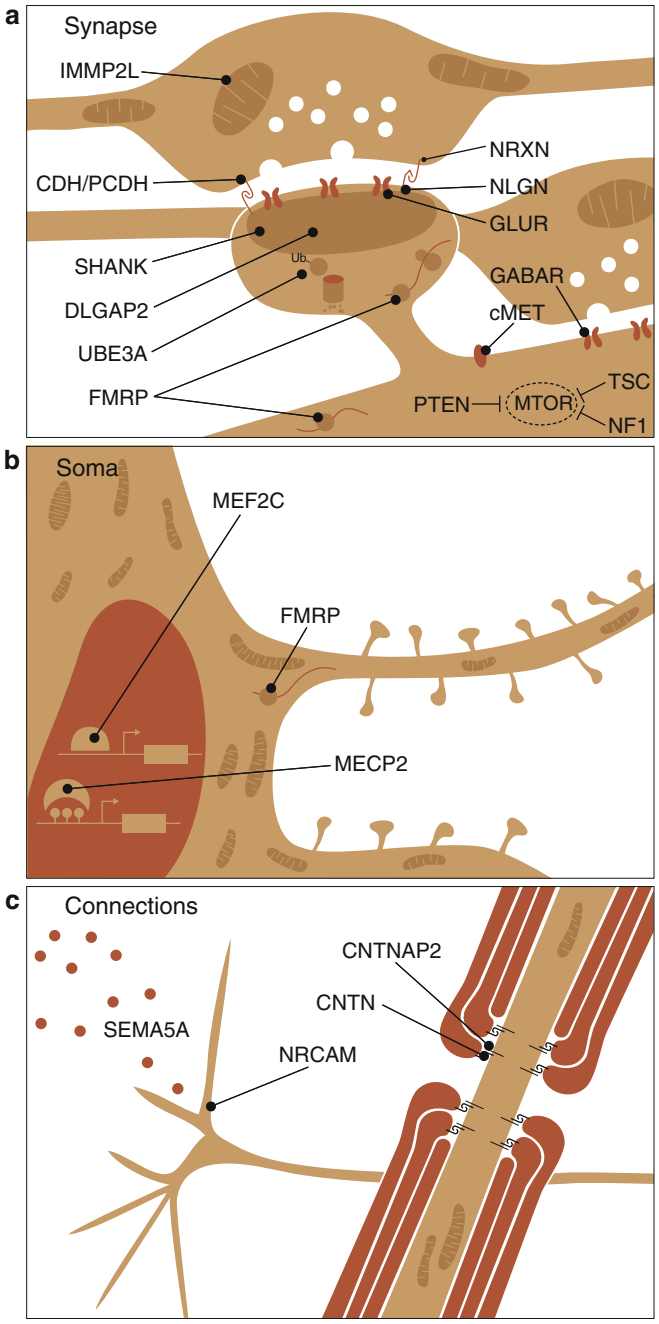


**Fig. 2 Biological processes involved in ASD**. The genes associated with ASD appear to participate in three main biological processes. First (panel A), at the synapse, cell adhesion proteins such as cadherins (CDH), protocadherins (PCDH), neuroligins (NLGN) and neurexins (NRXN), are

neurotransmitter. Children exposed *in utero* to VPA can display characteristic abnormalities grouped under the name "fetal valproate syndrome" and have an 8- to 18-fold increased risk of having ASD (Bromley et al. 2008; Chakrabarti and Fombonne 2001; Rasalam et al. 2005; Moore et al. 2000). The period of exposure to VPA that is likely to result in ASD is the first trimester of pregnancy (Arndt et al. 2005). In rat, a single exposure to VPA at an early stage of embryogenesis (during the period of neurulation, i.e., between E8 to E12.5) caused significant behavioral abnormalities in juvenile male offspring reminiscent of ASD. They displayed higher sensitivity to pain and lower sensitivity to nonpainful stimuli, stronger acoustic prepulse inhibition, lower locomotor, repetitive/stereotypic-like activity, and enhanced exploratory activity, decreased anxiety, increased number of social behaviors, and shorter latency to social explorations as well as disturbance of the circadian rhythm (Rodier et al. 1997; Schneider and Przewlocki 2005; Dufour-Rainfray et al. 2010; Tsujino et al. 2007). VPA is a direct inhibitor of histone deacetylase (HDAC; Phiel et al. 2001), an enzyme with an important role in the epigenetic regulation of gene expression. Using transcriptomics, Fukuchi et al. identified a set of genes differentially expressed after the treatment of cultured rat cortical neurons with VPA (Fukuchi et al. 2009). Among them, the BDNF and the α4 subunit of the GABAA receptor (GABAARα4) were up-regulated, whereas GABAARγ2, GAD65 and 67, and the K+/Cl − co-transporter KCC2, which are responsible for the development of GABAergic inhibitory neurons, were down-regulated. The number of GAD67-positive neurons decreased upon VPA treatment. The acetylation of histones H3 and H4 was increased in the promoters of up-regulated, but not down-regulated, genes. Thus, it was suggested that VPA might disrupt a balance between excitatory and inhibitory neuronal activities through its epigenetic effect (Fukuchi et al. 2009; Markram et al. 2008). Brain serotonin levels in VPA-exposed rats were also significantly higher than those in controls, suggesting that higher brain 5-HT levels might be responsible for irregular sleep/awake rhythm (Tsujino et al. 2007). Interestingly, environmental enrichment,

**Fig. 2** (continued) involved in synaptic recognition and assembly. Within the postsynaptic density, scaffold proteins such as SHANK3 and DLGAP2 assemble the synaptic components and provide a link between membrane proteins and the actin skeleton. FMRP transports mRNA at the dendrites and regulates local translation of synaptic proteins. In the cytoplasm, the mTOR pathway regulates translation and is influenced by proteins such as PTEN, NF1, TSC1/TS2 and c-MET. The E3 ligase UBE3A is involved in the targeting of synaptic proteins to the proteasome. Receptors for glutamate (GLUR) and GABA (GABAR) play a central role in producing excitatory and inhibitory currents, respectively. IMPP2L is a peptidase within the inner membrane of the mitochondria. Second (panel B), in the nucleus, the methyl binding protein MECP2 and transcription factors such as MEF2C regulate the expression of neuronal genes involved in the formation of neuronal circuits and synaptic functions. The FMRP protein transports and regulates the translation of mRNA at the synapse. Finally (panel C), at the nodes of Ranvier, proteins such as CNTN and CNTNAP2 organize the tight junctions between the axon and the myelinating glia. At the membrane or in the intercellular space, cell adhesion molecules and secreted proteins such as NRCAM or SEMA5A act as guidance cues for axonal outgrowth (Database list see at the end of the chapter)

a condition known to affect multiple aspects of brain function, seemed to counteract the behavioral alterations in VPA rats (Schneider et al. 2006).

## 4 Abnormal Level of Synaptic Proteins

Several lines of evidence indicate that genetic and/or epigenetic factors regulating synaptogenesis and neuronal circuit formation (Figs.1 and 2) are associated with an increased risk for ASD (Bourgeron 2009; Toro et al. 2010). Among these, several genes seem to regulate the level of proteins at the synapse. Two X-linked genes, *MeCP2* (see epigenetics in ASD) and *FMR1,* are involved in autism "secondary" to Rett and fragile X syndromes, respectively. FMRP (Fig. 2A, B) is a selective RNA-binding protein that transports mRNA into dendrites and regulates the local translation of some of these mRNAs at synapses in response to activation of metabotropic glutamate receptors (mGluRs). FMRP represses protein synthesis by forming a complex with the Cytoplasmic FMRP Interacting Protein 1 (CYFIP1) and the cap-binding protein eIF4E (Napoli et al. 2008; De Rubeis and Bagni 2011). In the absence of FMRP, an excess of mRNA translation alters synaptic plasticity (Kelleher and Bear 2008). Interestingly, both *CYFIP1* and *eIF4E* were also associated with ASD (van der Zwaag et al. 2010; Neves-Pereira et al. 2009).

Mutations of other genes associated with ASD seem to affect the level of synaptic proteins by dysregulating overall cellular translation (Kelleher and Bear 2008). Patients with neurofibromatosis, tuberous sclerosis, or Cowden/Lhermitte Duclos syndromes are at higher risk than the general population for ASD. These disorders are caused by dominant mutations in the tumor suppressor genes *NF1*, *TSC1*/*TSC2* and *PTEN* (Fig. 2). These genes code for proteins that act in a common pathway as negative effectors of the rapamycin-sensitive mTOR-raptor complex, a major regulator of mRNA translation and cellular growth in mitotic cells (Kelleher and Bear 2008). The mutations observed in ASD have been predicted to enhance the mTORC1 complex, which could lead to abnormal synaptic function due to an excess of protein synthesis. Interestingly loss of *Tsc1*/*Tsc2* or *Pten* in mice results in neuronal hypertrophy (Tavazoie et al. 2005), and patients presenting mutations in *NF1*, *TSC1*/*TSC2* and *PTEN* have a higher risk for macrocephaly. Further modulation of the PTEN and mTOR is exerted by serotonin and the proto-oncogene cMET, two pathways that are also associated with ASD (Cook and Leventhal 1996; Campbell et al. 2006).

Consistent with the hypothesis of a relationship between abnormal levels of synaptic proteins and ASD, many studies have reported mutations in genes involved in synaptic protein ubiquitination, including *UBE3A*, *PARK2*, *RFWD2* and *FBXO40* (Glessner et al. 2009; Fig. 2A). Ubiquitination proceeds through the ligation of ubiquitin to proteins, and this post-translational modification directs the ubiquitinilated proteins to cellular compartments or to degradation into the proteasome. The ligation of ubiquitin is reversible and could be used to regulate

specific protein levels at the synapse. In mice, many proteins of the post-synaptic density, including the ASD-associated SHANK proteins, are ubiquitinated in an activity-dependent homeostatic manner (Ehlers 2003). Ubiquitination involves activating enzymes (E1), conjugating enzymes (E2) and ligases (E3). Substrate specificity is usually provided by the E3 ligases, which typically have substrate-binding sites. UBE3A (also called E6-AP) is an E3 ligase encoded by an imprinted gene (only expressed from the maternal copy) and is responsible for Angelman syndrome (Schanen 2006). De novo maternal duplications of chromosome 15q11-q13 including *UBE3A* have been observed in 1-3 % of patients with ASD (Schanen 2006). It is still not clear whether *UBE3A* alone contributes to the risk of ASD, since other candidate genes are also duplicated on chromosome 15q11-q13; however, its role at the synapse has been recently demonstrated in mice (Dindot et al. 2008; Greer et al. 2010). In cultured hippocampal neurons, *Ube3a* is localized at the pre- and post-synaptic compartments but also at the nucleus. Neuronal activity induces *Ube3A* transcription, and then Ube3A regulates excitatory synapse development by controlling the degradation of Arc, a synaptic protein that promotes the internalization of the AMPA subtype of glutamate receptors (Greer et al. 2010). This process might have many consequences for synaptic structure, as suggested by Ube3a maternal-deficient mice, which exhibit abnormal dendritic spine development, including spine morphology, number and length (Dindot et al. 2008), and a reduced number of AMPA receptors at excitatory synapses (Greer et al. 2010).

Finally, the transcription factor *MEF2C* (Fig. 2B), which is involved in the regulation of the number of synapses, appears to be a risk factor for intellectual disability (Le Meur et al. 2010) and could therefore also be associated with ASD. Taken together, the genetic results obtained in humans and the functional studies mostly obtained in mice suggest that different independent mechanisms could alter the level of synaptic proteins; however, the actual nature of the impaired synaptic function(s) and its association with ASD phenotype remains to be characterized.

## 5 Abnormal Formation of Neuronal Circuits in ASD

The main category of genes associated with ASD is related to the development and the function of neuronal circuits (Bourgeron 2009; Toro et al. 2010). At the synaptic membranes, cell adhesion molecules, such as NLGNs and NRXNs (Fig. 2), are major organizers of excitatory glutamatergic and inhibitory GABAergic synapses and contribute to the activity-dependent formation of neuronal circuits in mice (Sudhof 2008). Mutations identified in patients with ASD were found to alter the ability of NLGNs to trigger synapse formation in cultured neuronal cells (Chih et al. 2004; Zhang et al. 2009). The disorders associated with NLGN-NRXN mutations can vary greatly among individuals, and this appears to be the case even for subjects of the same family, carrying the same mutation. Mutations of the X-linked *NLGN4X* have been associated with mental retardation (Laumonnier et al. 2004), typical autism (Zhang et al. 2009; Jamain et al. 2003), Asperger syndrome (Jamain

et al. 2003) and more recently with Tourette syndrome (Lawson-Yuen et al. 2008). In one case, a *NLGN4X* deletion was observed in a male with normal intelligence and apparently no autistic features (Macarov et al. 2007). *NRXN1*, by contrast, has been implicated in disorders such as schizophrenia and Pitt-Hopkins-like syndrome but has been also found in asymptomatic carriers (Zweier et al. 2009).

Interestingly, NLGN and NRXN might also play a role in social interaction in species other than humans without affecting overall cognitive functions. Mutant mice carrying a R451C *Nlgn3* mutation displayed an increased number of GABAergic synapses and inhibitory currents (Tabuchi et al. 2007), normal (Chadman et al. 2008) to reduced social interaction (Tabuchi et al. 2007) and a reduction of ultrasonic vocalization in pups (Chadman et al. 2008). The knockout mice for *Nlgn4* displayed reduced social interactions and ultrasonic vocalizations at the adult stage (Jamain et al. 2008). Accordingly, mutant knock-in *Nlgn3* and knock-out *Nlgn4* displayed enhanced to normal learning compared with wild-type mice (Tabuchi et al. 2007; Jamain et al. 2008). Furthermore, in the mouse model for fragile X, an enhanced Nlgn1 expression improved social behavior, whereas no effect on learning and memory was observed (Dahlhaus and El-Husseini 2010). Finally, in the honeybee, sensory deprived animals had a lower level of Nlgn1 expression but a generally increased level of the Nlgn2-5 and NrxnI expression compared with hive bees (Biswas et al. 2010).

The postsynaptic density plays a major role in the organization and plasticity of the synapse, and mutations affecting scaffolding proteins, such as SHANK2, SHANK3 and DLGAP2, are recurrently found in ASD (Durand et al. 2007; Moessner et al. 2007; Pinto et al. 2010). Deletions at 22q13 and mutations of *SHANK3* could be present in more than 1-2 % of ASD patients (Durand et al. 2007; Moessner et al. 2007; Gauthier et al. 2009). Shank proteins are a family of three members, which are crucial components of the postsynaptic density. Together with their binding partners, they have been shown *in vitro* to regulate the size and shape of dendritic spines (Roussignol et al. 2005). They also link glutamate receptors to the cytoskeleton, and variations in genes regulating cytoskeletal dynamics were associated with mental retardation and ASD (Pinto et al. 2010; Persico and Bourgeron 2006). Mice lacking *Shank3* display autism-like features including higher stereotypy, reduced social interactions and ultrasonic vocalizations (Bangash et al. 2011; Wang et al. 2011; Peça et al. 2011; Bozdagi et al. 2010). Mice lacking Shank2 also display reduced social interactions and ultrasonic vocalizations, but hyperactivity and anxiety (Schmeisser et al. 2012).

The role of neurotransmitter transporters and receptors in the susceptibility to ASD is still unclear. Because of the abnormally high levels of serotonin in ASD patients (Cook and Leventhal 1996), the serotonin transporter *SLC6A4* was extensively analyzed, and the results pointed toward dimensional rather than categorical roles for *SLC6A4* in stereotypic behaviors (Sutcliffe et al. 2005). For glutamate, only weak associations for *GRIK2* were detected (Jamain et al. 2002), and a duplication of the X-linked *GRIA3* receptor gene was observed in a patient presenting typical autism (Jacquemont et al. 2006). Concerning GABA, the most robust findings concern the duplication of the GABA receptor subunit gene-cluster

on chromosome 15q11-13 and the observation of maternal over-transmission of a rare variant of the GABA(A) receptor beta3 subunit gene (GABRB3; Delahanty et al. 2011; see above epigenetics and ASD).

Finally, proteins, related to axonal growth and synaptic identity are now also suspected to play a role in ASD. Semaphorins are membrane or secreted proteins (Fig. 2) that influence axon outgrowth and pruning, synaptogenesis and the density and maturation of dendritic spines. SNPs located close to the semaphorin *SEMA5A* were associated with ASD in a large cohort (Weiss et al. 2009). Independently, the level of *SEMA5A* mRNA was found to be lower in brain tissue and B-lymphoblastoid cell lines from patients with ASD compared with controls (Melin et al. 2006). The contactin family of proteins is involved in axonal guidance as well as in the connection between axons and glial cells, and ASD patients have been found to have deletions of the contactin genes *CNTN3* and *CNTN4* and the contactin associated protein *CNTNAP2* (Alarcón et al. 2008; Arking et al. 2008; Bakkaloglu et al. 2008; Roohi et al. 2009; Morrow et al. 2008). In addition, inherited CNVs or SNPs have been found in other cell-adhesion proteins – cadherins (*CDH9, CDH10, CDH18*) and protocadherins *PCDH9 and PCDH10* (Wang et al. 2009; Marshall et al. 2008; Morrow et al. 2008) – that might contribute to the susceptibility to ASD by altering neuronal identity.

## 6 Abnormal Synaptic Homeostasis in ASD

Based on the genetic/epigenetics and functional results, there is convergent evidence that abnormal synaptic homeostasis could be one of the causes of ASD. Different homeostatic mechanisms allow neuronal cells to maintain an optimal level of neuronal activity despite global changes in the overall activity of the network (Turrigiano 1999; Tononi and Cirelli 2003; Macleod and Zinsmaier 2006). Recent evidence suggests that homeostasis plays a role in the adaptation of synaptic plasticity by changing levels of activity (Ehlers 2003; Turrigiano 1999), and it might also be associated with the downscaling of synaptic weights during sleep (Tononi and Cirelli 2003). During development and the first years of life, activity plays an important role in the refinement of brain connections, and many results suggest that these processes are under homeostatic control at the synapse (Turrigiano and Nelson 2004). The genes and the mechanisms that we have surveyed in this chapter might disrupt synaptic homeostasis at various levels (Ramocki and Zoghbi 2008). The synthesis and degradation of different postsynaptic density proteins have been shown to vary as a function of activity (Ehlers 2003). Mutations in ubiquitin-dependent degradation could directly interfere with this process, as would also be the case if mutations were present in scaffolding genes such as the Shank family. Synaptic homeostasis has been shown to depend on local protein synthesis, on $Ca^{2+}$ concentration and on a tight regulation between the pre- and post-synaptic sides of the synaptic contact mediated by cell adhesion molecules such as NLGN and NRXN (Yu and Goda (2009). Finally, synaptic

homeostasis is not independent from cellular homeostasis and therefore should be affected by mutations altering gene expression level as well as neuronal numbers and shape, such as mutations related to the mTOR pathway.

If synaptic homeostasis is altered in ASD, environmental factors that influence this regulatory process could also modulate its severity. As reviewed elsewhere Bourgeron 2007; Cook and Leventhal 1996), abnormal serotonin and/or melatonin levels and altered sleep or circadian rhythms might constitute risk factors for ASD Melke et al. 2008). Sleep has been proposed as an important mechanism to regulate synaptic homeostasis. During wakefulness, there appears to be a global increase in the strength of excitatory synapses, which is scaled down during sleep to a baseline level (Tononi and Cirelli 2003; Miller 2009), a mechanism that can play an important role in learning and memory (Stickgold and Walker 2005). In addition to mutations of genes directly involved in synaptic processes, we have recently proposed that, in some cases, ASD could result from the interplay between abnormalities in synaptic and clock genes and that restoring circadian rhythms might therefore be beneficial for the patients and their families (Bourgeron 2007).

Most of the genes considered in this review are thought to be expressed throughout the brain; however, neuroimaging studies seem to converge into a stereotypical network of brain regions where differences between ASD and control populations can be detected. These two results would not need to be contradictory if different brain networks were differently resilient to variations in synaptic homeostasis. From an evolutionary standpoint, brain networks involved in more recently acquired cognitive skills, such as language or complex social behavior, might have fewer compensatory mechanisms compared with networks involved in more ancient biological functions that have been shaped by a much stronger selective pressure.

## 7 Concluding Remarks and Perspectives

It is just a matter of time before geneticists will obtain whole genome sequences of ASD patients. Exploring epigenetic alterations should also be more feasible in the near future, with the availability of brain tissue samples and stem cells from patients. Animal models based on genetic results are now under scrutiny in many laboratories and the consequences of the mutations and their reversibility are being analyzed from cell to behavior. However, more than ever we need to recognize the inherent heterogeneity of the genetic correlates of ASD. A true understanding of the relationship between genetic mutations and the ASD phenotype will not be possible if we persist in considering autism as a binary value in our analyses. Advancement in the research on ASD requires expertise from different fields, but only clinicians and psychiatrists will be able to determine what we are actually looking at (i.e., the autism phenotype or, rather, the different autism phenotypes). Future studies should tell us if increasing sample size or meta-analyses, phenotypic stratification, pathway analyses and SNP x SNP interactions can identify common

variants associated with sub groups of patients with ASD. Indeed, to date, it is not clear how many loci can regulate synaptic homeostasis and how these variants interact with each other to modulate the risk for ASD (Toro et al. 2010). A better knowledge of these genetic and epigenetic interactions will be necessary to understand the complex inheritance pattern of ASD.

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Autism CNV Database, http://projects.tcag.ca/autism_500k/
Autism Genetic Database (AGD), http://wren.bcf.ku.edu/
BioGPS, http://biogps.gnf.org/#goto=welcome
DECIPHER v4.3, https://decipher.sanger.ac.uk/application/.
UCSC Genome browser, http://genome.ucsc.edu

# Analysis of MeCP2 Function in the CNS

**Ege T. Kavalali and Lisa M. Monteggia**

**Abstract** Mutations in the methyl-CpG-binding protein (MeCP2) have been linked as the causative factor to Rett Syndrome (RTT). The *MECP2* gene encodes a DNA binding protein that binds to methylated cytosines in the mammalian genome. The disease-causing mutations found in RTT patients are predicted to result in the loss of function of MeCP2 that then results in genes turned on in an inappropriate manner. Over the past several years, our group has examined the role of MeCP2 in complex behavior as well as neurotransmission. Our behavioral analysis has shown that the forebrain-specific *Mecp2* loss-of-function in mice could recapitulate several behavioral and neurological deficits associated with RTT. Interestingly, we could also show that brain region-specific *Mecp2* loss-of-function, such as selective knock down of MeCP2 in basolateral amygdala, could trigger a subset of RTT phenotypes such as an increased anxiety-like behavior and deficits in cue-dependent fear conditioning. We have also demonstrated that loss of MeCP2 in neurons results in a decrease in spontaneous excitatory synaptic transmission coupled with an increase in action potential-driven excitatory drive. The combined effort of behavioral and synaptic analysis of MeCP2-associated phenotypes will not only open new avenues for understanding neuronal circuit abnormalities associated with neurodevelopmental disorders but also elucidate potential targets for addressing the pathophysiology of several intractable neuropsychiatric disorders.

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## 1 Introduction

Rett syndrome (RTT) is an X-linked neurodevelopmental disorder that has an incidence of 1:15,000 live female births and is one of the leading causes of mental retardation and autistic behavior in females (Hagberg et al. 1983). Individuals affected with RTT experience normal development up to the age of 6–18 months, at which time they fail to acquire new skills and enter a period of motor skill regression. With time, RTT symptoms become more pronounced and include a wide range of neurological defects including, but not limited to, stereotypical hand movements, seizures, mental retardation, autism-like behavior, sleep disturbances, problems with gait, and decelerated head growth. In addition, most children afflicted with RTT show a loss of social and cognitive abilities.

Mutations in the coding region of the methyl-CpG-binding protein 2 (*MECP2*) gene account for >96 % of classic RTT cases (Amir et al. 1999; Amir and Zoghbi 2000; Bienvenu et al. 2000; Huppke et al. 2000; Ravn et al. 2005; Wan et al. 1999). These mutations are predicted to result in loss of MeCP2 function (Ballestar et al. 2000; Yusufzai and Wolffe 2000). In addition to classic RTT, mutations in the *MECP2* gene have been identified in other patient populations, including Angelman Syndrome, autism, learning disabilities, and mental retardation syndromes (Carney et al. 2003; Chahrour and Zoghbi 2007; Lam et al. 2000; Watson et al. 2001; Ylisaukko-Oja et al. 2005; Zoghbi 2005). Duplication of the *MECP2* gene has also been detected in some male patients with mental retardation and progressive neurological symptoms (Van Esch et al. 2005). Collectively, these findings strongly suggest that alterations in MeCP2 expression or function contribute to disease progression with predominant neurological phenotypes and that the levels of MeCP2 expression must be tightly controlled under normal circumstances.

The *MECP2* gene encodes a DNA binding protein that interacts with methylated cytosines in genomes. However, compared to other methyl-CpG binding proteins, MeCP2 binding requires an enrichment of A/T bases adjacent to methyl-CpGs (Klose et al. 2005). Normally, MeCP2 is believed to act as a transcriptional repressor by binding to target gene promoters and silencing their transcription in many different tissues. However, a recent study suggests that MeCP2 may act as either a transcriptional activator or repressor in the hypothalamus (Chahrour et al. 2008). Another group using ChIP-chip analysis of a human neuroblastoma cell line, SH-SY5Y, found that MeCP2 interacts closely with promoters that are actively expressed (Yasui et al. 2007). Recent studies ascribe a more global role for MeCP2 in transcription via regulation of the chromatin state akin to histones (Skene et al. 2010, Cohen et al. 2011).

MeCP2 is expressed in many tissues; however, it is interesting that the majority of the RTT deficits are pronounced in the CNS. MeCP2 is expressed at high levels in the mammalian brain, in particular in neurons. Recent evidence suggests that MeCP2 may also be expressed at low levels in glia (Ballas et al. 2009). In immature neurons, MeCP2 expression is low but increases during neuronal maturation and reaches its highest level of expression in postmitotic neurons (Kishi and Macklis

2004; Matarazzo et al. 2004). This expression profile of MeCP2 during development may suggest that MeCP2 is involved in neuronal maturation and dendritic arborization (Francke 2006). Interestingly, this high level of MeCP2 expression in postmitotic neurons continues throughout adulthood, suggesting that MeCP2 may be a necessary factor for proper neuronal function in mature neurons. Therefore, it is possible that alterations in MeCP2 expression in specific brain regions is responsible for the behavioral abnormalities observed in individuals afflicted with RTT and related mental retardation syndromes.

## 2 Behavioral Characterization of Forebrain-Specific MeCP2 Loss-of-Function

Attempts to model the disease by generating constitutive *Mecp2* knockout (KO) mice results in the recapitulation of many of the neurological symptoms of RTT, although these mice die early in postnatal development (Chen et al. 2001; Guy et al. 2001; Tate et al. 1996). The early postnatal lethality of constitutive *Mecp2* KOs prevents their use in behavioral characterization studies. To circumvent these potential problems, we generated conditional KO mice, in which floxed *Mecp2* mice were crossed with calcium-calmodulin-dependent protein kinase II (CaMKII)-Cre transgenic mice to selectively delete *Mecp2* in forebrain neurons (Chen et al. 2001; Gemelli et al. 2006).

The conditional *Mecp2* knockout mice were indistinguishable from littermate controls by gross examination until approximately 16 weeks of age, at which time a weight phenotype, as well as sustained clasping of the hindlimbs, appeared in the KOs but not littermate controls (CTLs). The delayed emergence of phenotypes agrees with the neurodevelopmental characteristics of RTT. Since RTT patients suffer from motor impairments, we examined the conditional KO mice for motor coordination deficits. Hindlimb and forelimb clasping have been observed in several mouse models of motor dysfunction (Auerbach et al. 2001; Guidetti et al. 2001; van Dellen et al. 2001). Using the rotarod test to assess motor coordination, we found that the KOs performed significantly worse than littermate CTLs, even prior to the appearance of hindlimb clasping.

To more fully behaviorally characterize the conditional *Mecp2* KO mice, we used a wide array of behavioral paradigms (Gemelli et al. 2006). We found that the KOs had initial hypoactivity followed by normal locomotor activity. We examined whether the initial hypoactivity of the conditional *Mecp2* KOs in the locomotor activity paradigm was the result of altered anxiety-like behavior. Using the elevated plus maze and the open field test, we found that the *Mecp2* KOs had an increase in anxiety-related behavior, a phenotype that is commonly reported in RTT patients. We assessed learning and memory in the fear-conditioning paradigm. Context-dependent fear conditioning is a task that requires an intact hippocampus and amygdala, whereas cue-dependent fear conditioning is dependent on the amygdala (LeDoux 2000; Maren 2001). We found that the conditional *Mecp2* KOs had a slight trend towards a decrease in context-dependent fear conditioning and a

significant decrease in cue-dependent fear conditioning that was not due to a difference in pain sensitivity or responses to acoustic stimuli.

One of the most dehabilitating aspects of autism spectrum disorders is the social deficits. To examine the social interactions of the conditional *Mecp2* KO mice compared to littermate CTLs, we employed a two-trial social interaction test in which the amount of time the test animal spent in an interaction zone by itself (No Target), and when a C57BL/6 mouse (Target) was placed behind a wire mesh in the interaction zone, were calculated. We found that CTL mice showed a significant increase in the amount of time in the interaction zone when another mouse (Target) was present compared to the No Target baseline. In contrast, the conditional *Mecp2* KO mice showed no difference in time spent in the interaction zone whether another mouse was present or not, indicating no preference to interact with the Target mouse. In accordance with this finding, the conditional *Mecp2* KO mice also showed a significant increase in latency to enter the interaction zone when the Target mouse was present compared to the No Target session, whereas the CTL mice showed no difference between the two sessions. To confirm a social interaction deficit in the conditional *Mecp2* KO line, we employed another behavioral paradigm in which we assessed the ability of the conditional *Mecp2* KO mice to directly interact with another mouse. We placed either a conditional *Mecp2* KO or a littermate CTL mouse into an individual cage in the dark for 10 minutes to habituate. A four-week-old juvenile male C57BL/6 mouse was then placed into the cage of each experimental mouse. We used a juvenile mouse instead of an adult mouse because of its smaller size and less threatening appearance to the experimental animal. The amount of time the experimental animal interacted with the juvenile mouse over a two-minute period was determined. On the initial interaction, the conditional *Mecp2* KO showed a decreased trend in the amount of interaction with the juvenile mouse compared to littermate CTLs. We then tested the same experimental animal with the same juvenile mouse repeatedly for four consecutive days. At each day of testing, the conditional *Mecp2* KO mice interacted less with the juveniles compared to the CTL mice. On day 5, a novel juvenile C57BL/6 mouse was placed into each experimental cage and the amount of time the experimental mouse interacted with this juvenile was determined. The CTL mice showed a significant increase in the amount of time they interacted with the novel juvenile compared to the previous juvenile after four consecutive days of interaction. In contrast, the conditional *Mecp2* KO mice showed only a slight increase in the amount of time they interacted with the novel mouse compared to the previous juvenile animal, further suggesting a deficit in social interaction in this mouse line.

Collectively, these data suggest that the loss of MeCP2 selectively in neurons in the broad forebrain is sufficient to recapitulate aspects of RTT. The regional selectivity of the loss of MeCP2 likely explains why other behavioral features of RTT were not observed in the conditional KOs. A key finding of the study is that the *Mecp2* KOs displayed motor coordination and hindlimb clasping although MeCP2 was not deleted in the cerebellum, suggesting that motor coordination deficits are independent of cerebellum dysfunction. A second key finding is that the loss of MeCP2 in postnatal neurons was sufficient to produce behavioral features characteristic of RTT patients, suggesting that the phenotypes may not depend on

neurodevelopment per se but rather may be due to the role of MeCP2 in mature postmitotic neurons.

## 3 Behavioral impact of brain region-specific MeCP2 loss-of-function

While the data suggest that the general loss of *Mecp2* in the forebrain may underlie behavioral symptoms exhibited by RTT patients, it remains unclear which specific brain regions or neural pathways mediate these abnormalities. Given the importance of the basolateral amygdala (BLA) in the perception and modulation of emotion, including fear, stress and anxiety (Campeau and Davis 1995; Davis and Shi 2000; LeDoux 2000, 2007; Maren and Fanselow 1995; Rainnie et al. 2004; Roozendaal et al. 2002; Wilensky et al. 1999), we examined whether the loss of *Mecp2* in the BLA was sufficient for the development of some of the behavioral phenotypes exhibited in RTT patients (Adachi et al. 2009). The lack of a Cre mouse driver line specific for the amygdala posed a challenging quandary of how to selectively target this brain region. To this end, we utilized a virus-mediated approach to specifically delete *Mecp2* in the BLA.

We combined the adeno-associated virus (AAV) expression system with the Cre/loxP site-specific recombination system as previously described (Adachi et al. 2008). Floxed *Mecp2* mice were bilaterally injected with AAV expressing a fusion construct of Cre recombinase and GFP (AAV-CreGFP) to produce the regionally specific deletion, or as a control AAV expressing green fluorescent protein (AAV-GFP). Floxed *Mecp2* mice used in this study carry loxP sites flanking exon 3 of the Mecp2 gene, which encodes a majority of the methyl-DNA binding domain of MeCP2. Previous studies using this floxed *Mecp2* mouse line have demonstrated the efficient deletion of *Mecp2* upon expression of Cre recombinase (Chen et al. 2001; Gemelli et al. 2006). To determine the regional specificity of the viral placements, we used epifluorescence to confirm GFP expression in the BLA, followed by fluorescent in situ hybridization (FISH) to evaluate *Mecp2* expression. Moreover, we used a QRT-PCR approach to quantitate the reduction of *Mecp2* mRNA expression in the BLA and Western blot analysis to further validate the loss of MeCP2 expression following the viral injections.

Since MeCP2 has been suggested to be a transcriptional repressor, we examined whether the localized knockdown of *Mecp2* in the BLA would alter levels of acetylated histone H3 and histone H4, markers of active gene transcription. We found that the loss of *Mecp2* produced a significant increase in levels of acetylated H3, suggesting an upregulation of gene expression, similar to a previous report (Shahbazian et al 2002). However, we did not detect a change in H4 acetylation levels, suggesting that the loss of MeCP2 is mediating selective changes on chromatin.

To examine whether the localized knockdown of *Mecp2* in the BLA would recapitulate behavioral phenotypes observed in RTT patients, we tested these mice in several behavioral paradigms (Adachi et al. 2009). We found the selective loss of MeCP2 in the BLA resulted in mice with enhanced anxiety-related behaviors as

well as deficits in cue-dependent fear conditioning but with normal locomotor activity, motor coordination, and social interaction. These data suggest that previously observed social interaction deficits by the loss of *Mecp2* in the forebrain could be dissociated from the anxiety phenotype. Moreover, our findings suggest that MeCP2 in BLA normally plays an important role in certain types of learning and memory as well as anxiety behavior.

The use of conditional KO approaches to delete MeCP2 selectively in specific brain regions is becoming increasingly common. A recent study has utilized the Sim1 promoter to drive Cre expression during embryonic development in specific hypothalamic nuclei as well as the nucleus of the lateral olfactory tract of the amygdala. The Sim1 conditional *Mecp2* KOs were more aggressive, had abnormal elevated corticosterone serum levels in response to stress, and were hyperphagic and obese with normal metabolic rates (Fyffe et al. 2008). Another study utilized the tyrosine hydroxylase (TH) promoter to delete MeCP2 in dopaminergic and noradrenergic neurons. The TH conditional *Mecp2* KO mice had reduced dopamine and norepinephrine levels, were hypoactive and had impaired motor performance but had normal motor earning, anxiety-related behavior, social interactions and cognitive function (Samaco et al. 2009). To assess the loss of MeCP2 selectively in serotonin neurons, the Pet1 transcription factor that is necessary to restrict serotonergic neuronal phenotype has been used. The Pet1 conditional *Mecp2* KO mice have decreased serotonin levels in brain and increased aggression as assessed in the resident intruder paradigm, with no other reported behavioral phenotypes (Samaco et al. 2009). Recently, the vesicular inhibitory amino acid transporter, Viaat, has been used to delete MeCP2 globally in GABAergic neurons in the brain and spinal cord. The Viaat conditional *Mecp2* KOs recapitulated several of the behavioral features of RTT, including motor dysfunction, deficits in social behavior, impaired learning and memory, and excessive grooming behavior (Chao et al. 2010). Use of the Dlx5/6 promoter to delete MeCP2 in a subset of forebrain GABAeric neurons revealed a phenotype similar to that of the Viaat conditional *Mecp2* Kos, including impaired motor function and increased social behavior (Chao et al. 2010). However, the Dlx5/6 conditional MeCP2 KOs did not show any alterations in grooming behavior (RTT). The last two studies suggest a critical function of MeCP2 in GABAeric neurons in contributing to some of the behavioral phenotypes associated with RTT. Collectively, conditional KO approaches are revealing important and previously unknown roles for MeCP2 in distinct cell populations in the brain that may provide valuable clues for what underlies the disease pathophysiology.

## 4 Loss of MeCP2 Function Specifically Impairs Excitatory Neurotransmission

Since the loss of MeCP2 does not result in neurodegeneration, with only subtle neuroanatomical abnormalities, we wondered whether alterations in MeCP2 expression impact the functional properties of neurotransmission. To elucidate the role of MeCP2 in the regulation of synaptic transmission, we studied functional

alterations of synapses using hippocampal cultures made from newly born *Mecp2* constitutive KO mice. Previous studies using electrophysiological measurements of synaptic plasticity in *Mecp2*-deficient mice had revealed alterations on both hippocampal and cortical slices (Asaka et al. 2006; Dani et al. 2005). Dissociated primary cultures allow examination of synaptic function independent of potential general alterations in brain homeostasis, thus enabling a distinction between cell-autonomous defects and global systemic dysfunction. We examined spontaneous neurotransmission to see if the loss of MeCP2 impacted basal neurotransmission. We quantified the frequency and amplitude of spontaneous miniature excitatory postsynaptic currents (mEPSCs) in *Mecp2* KO and wild type littermate control (CTL) cultures using whole-cell recordings performed in the presence of tetrodotoxin (TTX) to block action potential firing and picrotoxin to block inhibitory activity. We found a significant decrease in the frequency of mEPSCs in the KO neurons compared to CTLs. The decrease was observed in young as well as older cultures, suggesting that the loss of MeCP2 produces long-term alterations in excitatory synaptic transmission. This alteration in mEPSC frequency may implicate a presynaptic deficit in the MeCP2 KO neurons. In contrast, the frequency of spontaneous miniature inhibitory postsynaptic currents (mIPSCs) was unaffected, suggesting a specificity of MeCP2 function in excitatory neurotransmission. The amplitudes of individual synaptic events in both mEPSCs and mIPSCs were also unaffected by the loss of MeCP2, indicating no potential change in the number of postsynaptic receptors at either excitatory or inhibitory synapses. To better understand how the loss of MeCP2 may contribute to the alteration in mEPSC frequency, we examined the number of presynaptic terminals formed on pyramidal neuron dendrites in culture and found no difference following the loss of MeCP2, suggesting that the decrease in spontaneous synaptic events is not the result of a decreased number of presynaptic terminals. Collectively, these data suggest that the loss of MeCP2 selectively impacts the excitatory/inhibitory balance in the brain, with a preference for a decrease in excitation, in agreement with other data showing a decrease in excitatory synaptic transmission between cortical as well as hippocampal neurons, with either no change or an increase in inhibition (Chao et al. 2007; Dani et al. 2005; Nelson et al. 2006; Tropea et al. 2009). These results are somewhat surprising, given that seizures occur in a majority of RTT patients, suggestive of cortical hyperexcitability (Glaze 2005).

To more closely examine this apparent disparity in excitatory-inhibitory balance, we have conducted a comprehensive analysis of short-term synaptic plasticity following the loss of MeCP2. Rather surprisingly, we found an increase in the ratio between excitation and inhibition in response to short-term, high frequency stimulation as well as evidence for enhanced excitatory presynaptic release probability in *Mecp2* KO hippocampal neurons (Nelson et al. 2011). Our data, taken together with the previous findings, indicate that basal, spontaneous activity in *Mecp2* KO neurons displays a decrease in excitation compared to inhibition, whereas evoked synaptic transmission shows an increase in excitatory drive. The disparate findings in excitation-inhibition balance in spontaneous and evoked transmission is surprising but not without precedent, and they suggest a distinct role for MeCP2

in regulation of basal neurotransmission and evoked neurotransmission (Atasoy et al. 2008; Fredj and Burrone 2009; Rothwell 2010; Sara et al. 2005; Sutton and Schuman 2009). Future studies will be necessary to further delineate how these alterations in neurotransmission may contribute to disease phenotypes.

## 5 Conclusion

The last two decades have witnessed significant advances in our understanding of gene regulation in neurons. In particular, synaptic activity has been shown to be a critical regulator of neuronal gene expression. A number of studies have focused on the role of synaptic N-methyl-d-aspartic acid (NMDA) receptors as well as dendritic L-type $Ca^{2+}$ channels and their downstream $Ca^{2+}$-dependent targets in the regulation of neuronal transcription within the nucleus. These processes have been shown to be essential for long-term neuronal adaptations and maintenance of synaptic plasticity. However, the impact of retrograde nuclear signaling and neuronal gene transcription on synaptic function has only recently begun to be elucidated. Over the past several years, there has been intense effort to delineate the role of MeCP2 in synaptic function as well as behavior. In this context, RTT syndrome and MeCP2-dependent neuronal gene regulation have provided a Rosetta stone for how alterations in transcription may impact neuronal function and, in turn, affect behavior. Studies from our group as well as others have shown that the function of this key epigenetic regulator strongly impacts synapse formation, maturation, and function. Despite the rapid pace of advances, the exact synaptic mechanisms and gene targets that mediate these effects on neurotransmission remain unclear. Moreover, these initial studies also highlight the importance of other key players and components of the MeCP2-dependent gene regulation pathway, which include histone deacetylases as well as DNA methyltransferases. These findings not only open new avenues for understanding neuronal circuit abnormalities associated with neurodevelopmental disorders but also elucidate potential targets for addressing the pathophysiology of several intractable neuropsychiatric disorders.

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# Epigenetic Mechanisms of Drug Addiction

**Ian Maze and Eric J. Nestler**

**Abstract** Drug-induced alterations in gene expression within the reward circuitry of the brain are thought to contribute importantly to the persistence of the drug-addicted state. Recent studies examining the molecular mechanisms by which repeated administration of drugs of abuse induces transcriptional changes have demonstrated a key role for chromatin remodeling and have directly related such chromatin regulation to the promulgation of addictive behaviors. In this review, we discuss recent advances in our understanding of chromatin phenomena—which can be referred to as epigenetics—that contribute to drug addiction, with the goal that such mechanistic insights will aid in the development of novel therapeutics for future treatments of addiction.

## 1 Introduction

Drug addiction is a debilitating chronic relapsing disorder that is characterized by pathological drug seeking and taking despite severe adverse consequences (Kalivas et al. 2005; Hyman et al. 2006; Koob and Kreek 2007). Addiction to several drugs of abuse has been shown to be 40-60% genetic; however, the specific genes that comprise the large majority of this risk remain unknown (Baker et al. 2011). Studies of the remaining ~50% of the risk for addiction have focused on the environmental components of drug addiction, for example, exposure to physical or emotional abuse, crime, and poverty, which are much more difficult to decipher

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mechanistically and remain vaguely defined. The central hypothesis in the field is that exposure to a wide range of environmental stimuli, which interact with an individual's genetic constitution, determines initial responses to drugs of abuse, as well as maladaptations to repeated drug exposure that underlie the transition to an addicted state. Because the behavioral abnormalities that characterize addiction are so long-lived, the field has focused on drug-induced changes in gene expression, which are believed to be generally more stable than other modes of cell regulation, such as allosteric and covalent modifications of proteins (Nestler 2001).

The goal of this work has been to characterize drug regulation of gene expression in addiction-related brain regions. Most focus to date has been on the nucleus accumbens (NAc), which normally controls responses to natural rewards (e.g., food, sex, social interaction) but is corrupted by drugs of abuse to help drive the addicted state. It is within this framework that investigators have, over the past several years, begun to focus on epigenetic mechanisms of drug addiction.

## 2 Modes of Epigenetic Regulation

The term epigenetics was coined decades ago to signify alterations in gene expression that occur independently of a change in DNA sequence (Bird 2007). These changes occur through highly complex alterations in the packaging of DNA—i.e., alterations in chromatin structure—within the cell nucleus. Such epigenetic regulation involves the covalent modifications of histone proteins (e.g., acetylation, methylation, and phosphorylation, among many others), methylation of DNA itself, and the concerted actions of literally hundreds of chromatin regulatory proteins (Jenuwein and Allis 2001; Jaenisch and Bird 2003; Berger 2007; Borrelli et al. 2008).

The term epigenetics is currently used very broadly to indicate several very different modes of regulation. First, in the context of drug addiction, epigenetics represents the molecular mechanisms by which repeated exposure to a drug of abuse causes altered gene expression in discrete brain regions. Many, perhaps even most, of these changes are short-lived, with only a small subset persisting for long periods of time to help sustain an addiction. Studies at the epigenetic level are providing the first ever glimpse of the detailed transcriptional mechanisms by which drugs of abuse alter the expression of a given gene within the brain in vivo. This approach is in contrast to all prior approaches, which relied by necessity on in vitro assays for mechanistic information despite the fact that what occurs in cultured cells, even in cultured neurons, is not an accurate reflection of what occurs in the brain. Similarly, exposure to a host of environmental factors would modify an individual's vulnerability or resistance for addiction by epigenetic-based alterations in gene expression in specific brain regions. A subset of such changes that occur early in development is likely very stable and presumably helps to set a person's risk for addiction for the lifetime of that individual.

A third type of epigenetic regulation occurs during brain development, as small numbers of neural stem cells give rise to billions of highly differentiated neurons and their trillions of synaptic connections. Much of this selective unfolding of gene expression patterns occurs as part of complex developmental programs, but a

portion is believed to arise stochastically as randomly occurring differences in gene expression that help to determine a range of brain functions for a lifetime. Such stochastic events explain, for example, why identical twins have very different patterns of cerebral gyri despite identical genes.

Finally, there has been recent interest in the theoretical possibility that drug exposure might induce epigenetic modifications in germ cells, which are somehow passed onto offspring to influence addiction vulnerability. It is certainly conceivable that systemic exposure to high levels of a drug of abuse (typical concentrations seen clinically are mM for alcohol, mM for other drugs) causes chromatin modifications in sperm and egg cells. However, it remains far-fetched that such changes would persist in offspring and drive changes in brain gene expression that would help set an individual's risk for addiction. Whether epigenetic transmission occurs is ultimately an empirical question, but one that has not yet been demonstrated convincingly.

To date, virtually all studies of epigenetic mechanisms in the drug addiction field have focused on drug-induced changes in chromatin structure within the NAc and other regions of the adult brain; much further work is required to examine epigenetic regulation occurring earlier in life. Here, we summarize this research, with a focus on epigenetic regulation by cocaine and related psychostimulants on which the vast majority of research has thus far focused.

## 3 Epigenetic Regulation by Cocaine

### *3.1 Epigenetic Modification of Drug-Regulated Genes*

Acute or repeated exposure to cocaine or amphetamine has been shown over the last few years to induce alterations in histone acetylation, methylation, or phosphorylation at numerous genes in the NAc and, to a lesser extent, in other limbic brain regions of rats and mice. These modifications are being related increasingly to specific forms of drug-induced neural plasticity within this reward circuitry and to drug-induced behavioral abnormalities that model human addiction (Renthal and Nestler 2008; LaPlant and Nestler 2011).

For example, by using quantitative immunoprecipitation (qChiP) assays, acute cocaine administration was shown to rapidly (i.e., effects appear within 30 minutes of drug treatment) and transiently (i.e., effects disappear by three hours following drug exposure) increase histone H4 acetylation at the proximal promoters of the immediate early genes *c-fos* and *fosb* in the NAc, a time course that is consistent with their kinetics of induction by cocaine (Kumar et al. 2005). Interestingly, although several control gene promoters were unaffected by acute cocaine (e.g., β-tubulin and histone H4), total tissue levels of H4 acetylation and H3 phosphoacetylation, as determined by Western blotting or immunohistochemistry, were transiently increased by drug treatment, suggesting that global alterations in histone

acetylation may act to influence the transcriptional profiles of a subset of genes involved in mediating cocaine-induced behaviors (Brami-Cherrier et al. 2005; Kumar et al. 2005). Cocaine also induces global changes in di- and trimethylation of Lys9 of histone H3 in NAc, as will be discussed below. Global changes in histone marks have also been observed in the prefrontal cortex (PFC) of rats in response to adolescent cocaine exposure, in which both the activating mark, trimethylation of Lys4 of H3 (H3K4me3), and the repressive mark, trimethylation of Lys27 of H3 (H3K27me3), were reduced (Black et al. 2006). The significance of such global changes remains unknown, since more recent genome-wide analyses have shown many gene promoters that display histone modifications in the opposite directions of such global alterations (see below).

Unlike acute drug exposure, chronic cocaine, either experimenter-delivered or self-administered, has been found to persistently induce expression of a distinct set of genes in the NAc (e.g., *bdnf* and *cdk5*; Bibb et al. 2001; Grimm et al. 2003). In line with such stable changes in gene expression, levels of H3 acetylation increased at the promoters of both *cdk5* and *bdnf* for one to seven days following drug exposure (Kumar et al. 2005). Stable alterations in acetylation and gene expression have also been demonstrated to occur in the PFC following relatively long periods of withdrawal from cocaine self-administration (Freeman et al. 2008).

Following initial studies aimed at analyzing the effects of drug exposure on chromatin regulation at specific gene promoters via qChIP, it was important to utilize genome-wide techniques to characterize such drug-induced histone modifications across every gene promoter throughout the genome. To do so, chromatin that was selectively immunoprecipitated using antibodies directed against polyacetylated H3 or H4, or methylated H3 (H3K9me2 and H3K27me2), was hybridized to genome-wide promoter microarrays (ChIP-chip). These experiments allowed us to compare enrichment of these marks at promoters across the genome from the NAc of cocaine-naïve vs. cocaine-experienced animals (Fig. 1A and B; Renthal et al. 2009). Genomic binding patterns of the drug-induced transcription factors CREB and ΔFosB (Nestler 2001, 2008) were also assessed and compared to drug-induced chromatin modifications to better identify putative target genes that may mediate persistent behavioral syndromes following chronic drug exposure (e.g., Fig. 1C). These high-resolution genomic maps of histone modifications and transcription factor binding patterns provide new insight into the function of such events in the in vivo regulation of neuron-specific transcriptional responses to drugs of abuse.

For example, these studies directly addressed earlier findings indicating that acute cocaine treatment results in increased H4 acetylation at acutely regulated gene promoters, whereas H3 acetylation appeared to predominate at chronically induced promoters (Kumar et al. 2005). ChIP-chip analyses revealed that many more genes display hyperacetylation on H3 in comparison to H4 (Fig. 1A and B), consistent with previous reports. Numerous previously unidentified promoters were also shown to be hyperacetylated at H4 following chronic cocaine treatment, indicating that both of these marks are likely involved in repeated cocaine-induced gene transcription (Renthal et al. 2009). Interestingly, only a very small subset of gene promoters displayed hyperacetylation at both H3 and H4 following chronic

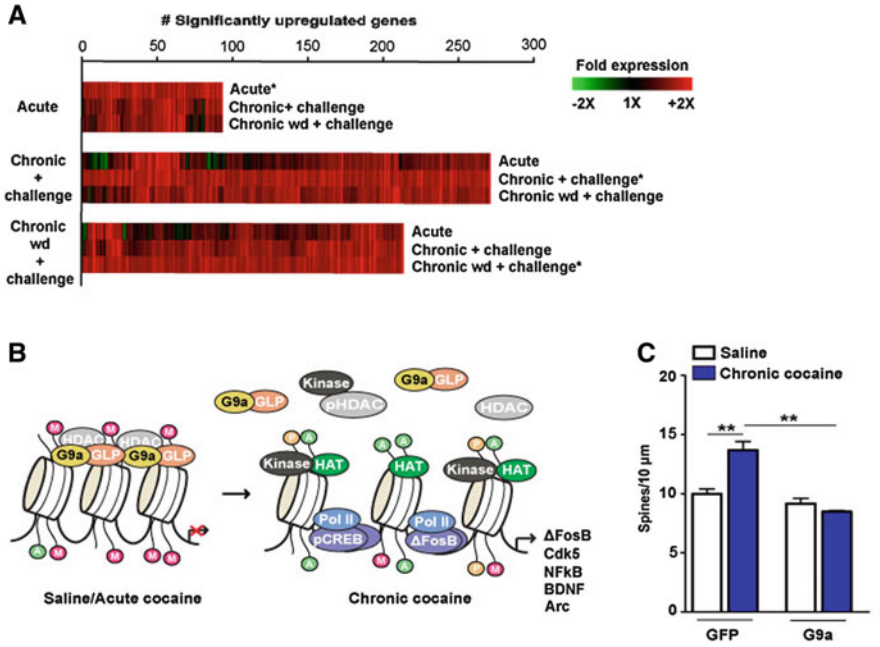


**Fig. 1** Chronic cocaine alters genome-wide patterns of histone modifications and transcription factor binding in NAc. (A) Venn diagrams and (B) heat maps depicting genes displaying altered levels of H3 or H4 acetylation and H3 methylation (dimethyl-K9/K27) promoter occupancy 24 hr after chronic cocaine administration. (C) Venn diagrams of genes displaying significant promoter binding of the transcription factor ΔFosB, and/or of H3/H4 acetylation or H3 methylation in NAc, following chronic cocaine administration. Adapted from Renthal et al. (2009) with permission

cocaine administration, suggesting that, although both modifications may be important for cocaine's effects on gene expression, the two marks most likely function independently on distinct subsets of genes to promote differential downstream effects. Another interesting discovery from these data was the finding that a very limited number of genes displayed hypoacetylation on either H3 or H4 after chronic cocaine. These data suggest that, in general, cocaine acts to enhance patterns of gene expression in the NAc through active acetylation, or inhibition of deacetylation, to promote transcriptional plasticity and expression of numerous drug-related transcripts, some of which may be important in the development of cocaine-induced behaviors (Renthal et al. 2009).

Renthal et al. (2009) also demonstrated that there was very little overlap between genes that displayed altered H3/H4 acetylation vs. H3K9/K27me2 (Fig. 2A and B). These results suggest that these two marks may mediate transcriptional events at largely distinct subsets of genes. Also, while global levels of H3K9me2 are downregulated by cocaine as stated earlier (Maze et al., 2010), large numbers of genes exhibited increased levels of this mark (Renthal et al. 2009), demonstrating the importance of genome-wide methods to establish the chromatin state of individual genes.

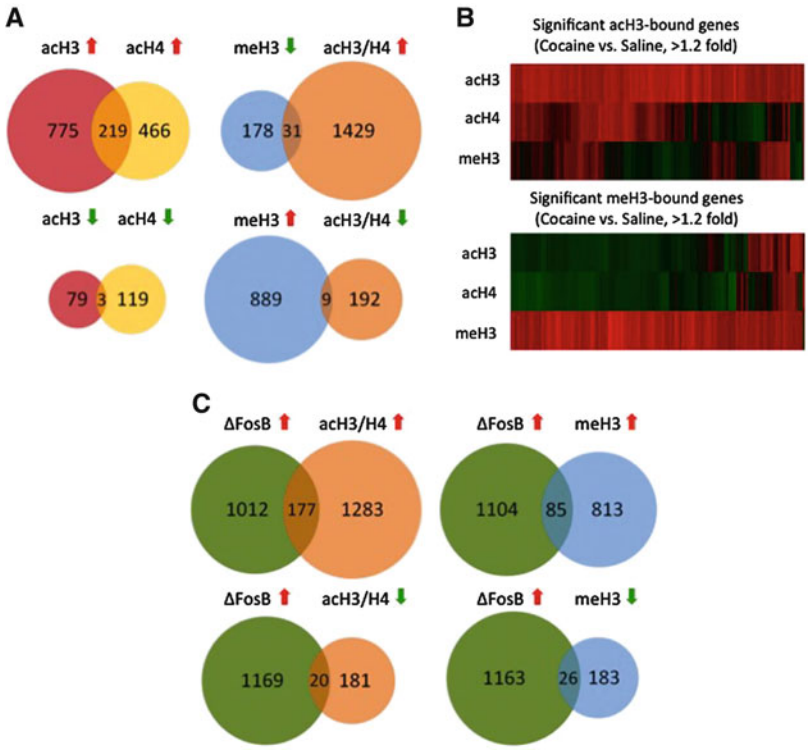


**Fig. 2** Chronic cocaine administration increases both transcriptional and structural plasticity in NAc through chromatin-dependent mechanisms. (A) Analysis of gene expression after acute or repeated cocaine indicates increased transcriptional activation/inducibility in NAc. Heat maps (*) display genes up-regulated in NAc 1 hour after a cocaine challenge in naïve animals (acute), in animals treated repeatedly with cocaine (chronic + challenge), or in animals after seven days of withdrawal from chronic cocaine (chronic wd + challenge). Associated heat maps show how genes are affected under the other two conditions. (B) Methylation of H3K9me2 promotes chromatin condensation and gene repression. Following either saline or acute cocaine exposure, a complex of histone methyltransferases (HMTs), mainly G9a and GLP, along with HDACs (e.g., HDAC5) binds to histones and repress transcription. After chronic cocaine administration, however, repressive HMT and HDAC binding to plasticity-related gene promoters is reduced, which, in combination with increased kinase activity and binding of histone acetyltransferases (HATs), ΔFosB, phosphoCREB, and RNA polymerase lI (Pol II) results in a permissive state of transcription. Such decreased binding of repressive chromatin complexes results in increased structural plasticity on NAc medium spiny neurons. (C) Overexpression of G9a in NAc, which opposes the endogenous effects of cocaine, blocks cocaine-induced alterations in transcription and significantly reduces structural plasticity in this brain region. Adapted from Maze et al. (2010) and Maze and Russo (2010) with permission

More recently, cocaine was shown to dynamically regulate global levels of H3K9me3 in the NAc, with acute cocaine increasing levels of this mark and chronic cocaine causing a persisting decrease (Maze et al. 2011). This finding was surprising since H3K9me3 is a marker of highly condensed heterochromatin,

which is not thought to be highly regulated in fully differentiated adult neurons. Moreover, the chronic cocaine-induced decrease in H3K9me3 is associated with reduced levels of other markers of heterochromatin and with an increase in the size of individual nuclei of NAc neurons, consistent with an overall increase in the permissive state of gene regulation induced by chronic cocaine. ChIP for this mark, followed by next-generation sequencing (ChIP-Seq), demonstrated that virtually all H3K9me3 binding occurs, as expected, in non-genic heterochromatic regions, including at specific repeat elements, some of which are induced in NAc by chronic cocaine. These findings reveal novel mechanisms governing the many ways in which repeated exposure to cocaine alters the function of NAc neurons and thereby induces a state of addiction.

Taken together, these types of analyses are allowing for a much better understanding of the genomic chromatin signatures that result from chronic treatment with cocaine or other drugs of abuse. Such analyses, as applied increasingly to several brain reward regions, will aid in the identification of gene targets that may be important in regulating diverse aspects of addictive behaviors.

## *3.2 Cocaine Regulation of Chromatin Modifying Enzymes: Histone Acetylation and Phosphorylation*

Some of the first evidence to suggest a role for epigenetic phenomena in the development of addictive-like behaviors came from studies demonstrating that pharmacological inhibition of histone deacetylases (HDACs), or genetic manipulation of class IIa HDACs, dramatically affected animals' behavioral responses to cocaine. Systemic administration of two non-specific HDAC inhibitors (HDACis), sodium butyrate (NaBut) or trichostatin A (TSA), as well as intra-NAc delivery of suberoylanilide hydroxamic acid (SAHA), a more specific HDACi, significantly potentiated cocaine's behavioral effects as determined by the conditioned place preference (CPP) measure of drug reward, as well as by locomotor responses to the drug (Kumar et al. 2005; Renthal et al. 2007). Similar effects of HDACis on locomotor sensitization can be observed with dopamine D1-specific agonists, amphetamine, morphine, or ethanol (Kalda et al. 2007; Schroeder et al. 2008; Sanchis-Segura et al. 2009). Interestingly, recent studies suggest that HDAC inhibition potentiates extinction from CPP and reduces reinstatement to this behavior (Malvaez et al. 2010; McQuown and Wood 2010). HDAC inhibition, and increased acetylation, may thus generally function to enhance behavioral saliency and promote adaptive responses to a large variety of environmental stimuli. Consistent with these findings, mice that are genetically depleted of the histone acetyltransferase, CREB-binding protein (CBP), exhibited reduced behavioral sensitivity to cocaine and displayed reduced acetylation at the *fosb* promoter (Levine et al. 2005).

Reducing acetylation through viral-mediated overexpression of specific HDACs in the NAc (i.e., HDAC4 and 5, but not HDAC9) decreased CPP, an effect that depended on the catalytic activity of the enzymes (Renthal et al. 2007). On the other hand, mice globally lacking HDAC5 exhibited enhanced rewarding responses to

cocaine, and this effect was rescued by targeted overexpression of this enzyme in the NAc. The finding that certain HDACs, including HDAC5, may specifically control aspects of drug-induced behavior prompted further investigations into the mechanisms through which cocaine may exert its effects on histone acetylation following drug exposure. It was observed that chronic, but not acute, exposure to cocaine induced HDAC5 phosphorylation and nuclear export of the enzyme via the chaperone protein 14-3-3 (Renthal et al. 2007). This event requires the enzymatic activity of CaMKIIa, which has also been demonstrated to be epigenetically and transcriptionally induced by psychostimulant exposure (Anderson et al. 2008; Loweth et al. 2010). These events ultimately result in hyperacetylation and increased expression of HDAC5 target genes in the NAc, phenomena that have also been observed in animals' chronically self-administering cocaine (Host et al. 2009).

It should be noted that, although these biochemical changes occur in animals receiving both experimenter-delivered and self-administered cocaine, systemic HDAC inhibition promotes differential behavioral responses to cocaine in self-administration paradigms. These effects depend on the time at which HDACi treatment is initiated. For example, rats that are treated with the HDACi, TSA, before each fixed ratio 1 scheduled self-administration session displayed significantly reduced numbers of cocaine self-injections during subsequent training sessions, indicating that these animals find cocaine to be less rewarding in this reinforcement paradigm (Romieu et al. 2008). In contrast, animals that are trained to self-administer cocaine prior to HDAC inhibition with the non-specific HDACi, NaBut, displayed increased lever pressing for cocaine, suggesting that these animals find cocaine to be more reinforcing. This result is consistent with data indicating enhanced behavioral responses to drugs of abuse following HDACi treatment (Sun et al. 2008). Similar results were observed in rats receiving daily intra-NAc infusions of SAHA, where HDAC inhibition caused an upward shift in the dose response curve under a fixed-ratio schedule and increased the break point under a progressive-ratio schedule, indicating enhanced motivation for self-administered drug (Wang et al. 2010). Further work is needed to understand the basis of these inconsistent findings.

The class III HDACs, sirtuin 1 and sirtuin 2 (SIRT1 and SIRT2), were identified by genome-wide ChIP-chip analyses as potential targets of increased H3 acetylation after chronic cocaine administration. Both genes were transcriptionally induced by the drug, leading to their increased enzymatic activity in the NAc (Renthal et al. 2009). Further examination of these genes' promoters revealed a possible role for the transcription factor ΔFosB in the regulation of sirtuin expression and a potential function for these enzymes in the development of cocaine-induced behaviors. Pharmacological manipulation of sirtuin activity through administration of the sirtuin activator, resveratrol, or the sirtuin inhibitor, sirtinol, produced opposite effects on cocaine-induced behaviors. Sirtuin inhibition decreased CPP for the drug and decreased cocaine-self administration, whereas sirtuin activation enhanced drug responses (Renthal et al. 2009). Current work is focused on the targets for SIRT1 and SIRT2, in addition to histones, that are responsible for these behavioral actions.

Histone H3 phosphorylation and phospho-acetylation, which are considered to be activating marks of transcription, have also recently been shown to be important

in the development of cocaine-induced behaviors. For example, cocaine administration rapidly induces H3 phosphorylation and phospho-acetylation in the striatum (Brami-Cherrier et al. 2005; Kumar et al. 2005). The H3 kinase MSK1, a known downstream member of the MAP kinase signaling cascade, was demonstrated to mediate this cocaine-induced increase in H3 phosphorylation (Brami-Cherrier et al. 2005). Furthermore, depletion of MSK1 results in reduced locomotor responses to the drug, consistent with a role for histone phosphorylation events in mediating drug-induced behavioral sensitivity. The protein phosphatase inhibitor, DARPP32, has also been shown to regulate changes in histone phosphorylation in striatum in response to cocaine administration (Stipanovich et al. 2008).

Corresponding data suggest that loss of MSK1 blocks the acute induction of the immediate early gene *c-fos* (Brami-Cherrier et al. 2005). This acute induction of *c-fos* can be potentiated by HDACis. However, chronic psychostimulant administration has been shown to desensitize *c-fos* expression, an event that results, in part, from recruitment of a co-repressive ΔFosB:HDAC1 (class I HDAC) complex to the *c-fos* promoter (Kumar et al. 2005; Renthal et al. 2008). Current research is focusing on how these multiple modes of regulation of *c-fos* and perhaps other genes are integrated to mediate the complex behavioral actions of cocaine.

### *3.3 Cocaine Regulation of Chromatin-Modifying Enzymes: Histone Methylation*

Although ample data have related histone acetylation and phosphorylation to the regulation of drug-induced behaviors, only more recently has the considerably more stable modification, histone methylation, been demonstrated to be an essential mediator of drug-induced transcriptional, synaptic, and behavioral plasticity (Maze et al. 2010). As previously mentioned, genome-wide ChIP-chip analyses revealed that the specific histone methylation marks, H3K9me2 and H3K27me2, displayed altered binding at numerous gene promoters in NAc after chronic cocaine (Renthal et al. 2009). To further investigate these findings, a transcriptional profiling approach was taken to ask if expression of specific histone methylating or demethylating enzymes was regulated in this region following repeated cocaine treatment. Of all of the enzymes examined, only two, G9a (EHMT2) and GLP (EHMT1), displayed persistent regulation by repeated experimenter-delivered and self-administered cocaine. Both enzymes were significantly downregulated in the NAc, consistent with the global decreases in levels of H3K9me2 in this brain region as noted earlier (Maze et al. 2010). This mark is associated with transcriptional repression and is found primarily in euchromatic regions of the genome.

While many cocaine-induced alterations in gene expression appear transiently after drug exposure, numerous genes display enhanced transcriptional inducibility (i.e., sensitization) following repeated drug administration (Fig. 2A). This phenomenon is largely dependent on downregulation of G9a activity in the NAc (Maze et al. 2010). Thus, as G9a is repressed following chronic cocaine administration, G9a

target genes become more sensitive (i.e., more likely to be transcriptionally induced) by future exposures to the drug (Fig. 2B). Furthermore, NAc-specific knockout of G9a, which mimics the effects of chronic cocaine exposure, enhances drug-associated reward behaviors. On the other hand, viral-mediated overexpression of G9a blunts this response, an event that has been shown to be dependent on G9a's catalytic activity (Maze et al. 2010).

To identify potential mechanisms through which G9a is repressed by chronic cocaine, as well as to identify putative G9a target genes regulated in response to cocaine treatment, a negative feedback loop was identified. In response to acute cocaine, G9a acts to maintain homeostatic levels of gene expression in the NAc by repressing genes known to enhance synaptic plasticity in this brain region. However, following chronic treatment with the drug, G9a recruitment to target gene promoters is diminished, leading to the enhanced transcriptional inducibility/expression of associated transcripts (Maze et al. 2010). This process was demonstrated to be dependent on ΔFosB accumulation following chronic cocaine treatment. Acute exposure to cocaine dramatically increased G9a binding to the *fosb* promoter, thus rapidly repressing cocaine-induced increases in ΔFosB expression. Following repeated cocaine exposure, however, ΔFosB accumulation in the NAc results in G9a repression and reduced global levels of H3K9me2, preventing G9a's ability to maintain normal levels of gene expression and enhancing behavioral responses to the drug (see Fig. 2B for schematic; Maze et al. 2010). Such inability to repress synaptic plasticity-related gene expression following chronic cocaine administration can result in enhanced dendritic spine plasticity in the NAc, an event that generally correlates with increased behavioral sensitivity to the drug (Robinson and Kolb 2004; Russo et al. 2010) and can be rescued by G9a overexpression in this brain region (Fig. 2C; Maze et al. 2010). Interestingly, ΔFosB overexpression in the NAc, in the absence of cocaine, is sufficient to reduce G9a recruitment to target gene promoters and promotes increased dendritic spine density in this brain region, whereas blockade of ΔFosB function prevents the ability of cocaine to induce NAc dendritic spines. These findings marked some of the first evidence of a role for chromatin regulation in the structural/morphological changes observed following repeated cocaine exposure, likely contributing to enhancements in drug-induced behavioral sensitivity.

### 3.4 *Cocaine Regulation of Chromatin-Modifying Enzymes: DNA Methylation*

Surprisingly, given the stability of the modification and its potential in mediating drug-induced transcriptional responses, very little is known concerning the role of DNA methylation in the development of persistent addictive-like behaviors. Recent studies examining global patterns of DNA methylation in the hippocampus of neonatal and prepubertal animals following in utero cocaine exposure indicate that altered levels of DNA methylation in this brain region may be important in regulating persistent patterns of drug-induced gene expression, which may

ultimately contribute to enhanced behavioral sensitivity later in life (Novikova et al. 2008). In accordance with these findings, the methylated DNA binding protein, MeCP2, has also been shown to be induced in cortical regions, as well as in the striatum, following chronic cocaine self-administration, correlating with changes in gene expression observed post-drug treatment (Host et al. 2009). Cocaine or amphetamine regulation of MeCP2 in NAc, or in dorsal striatum, has been linked directly with altered behavioral responses to the drug, although the specific action seems to differ in these two brain regions (Deng et al. 2010; Im et al. 2010).

A direct role for the DNA methylating enzyme, DNA methyltransferase 3a (DNMT3a), in cocaine action has been demonstrated as well. Reduced DNMT3a activity in the NAc, either by pharmacological inhibition or via viral-mediated knockdown of the enzyme, results in increased behavioral responses to cocaine (LaPlant et al. 2010). Since DNMT3a generally functions to repress gene transcription, these data further support the hypothesis that aberrant increases in gene expression following repeated cocaine administration promote increased behavioral sensitivity to the drug. Interestingly, however, DNMT3a, unlike the G9a (Maze et al. 2010), promotes increased dendritic spine density in the NAc following cocaine administration (LaPlant et al. 2010). Such paradoxical findings likely reflect "homeostatic adaptations" that compensate for additional alterations caused by repeated cocaine exposure, such as a reduction in glutamatergic stimulation of NAc neurons by prefrontal cortical afferents (Thomas et al. 2001; Berglind et al. 2009). On the other hand, given that DNMT3a overexpression alone was sufficient to enhance NAc spine density, in combination with observations that these changes were not potentiated further by cocaine exposure, it is possible that such overexpression results in a form of metaplasticity in which increased expression of DNMT3a in this brain region promotes elevated structural plasticity, thus superseding additional alterations in spine density resulting from chronic cocaine administration. These data, as well as other examples of proteins known to increase NAc dendritic spine density but reduce behavioral responses to cocaine (e.g., CDK5 and MEF2), highlight the complexity of these intracellular pathways and the importance of future research (Bibb et al. 2001; Norrholm et al. 2003; Pulipparacharuvil et al. 2008; Russo et al. 2010).

Despite this evidence for DNA methylation controlling neural and behavioral plasticity to cocaine, very little is known about the specific genes involved. An important need for the field is to use any of several methods of measuring DNA methylation profiles at specific genes in a genome-wide fashion in cocaine models and to directly link altered methylation of a given gene with cocaine-induced plasticity.

## 4 Future Research and Conclusions

Remarkable progress has been made in documenting distinct regulatory patterns of chromatin modifications and the subsequent activity of transcription factors and their downstream targets in response to administration of several drugs of abuse. However, many important questions remain regarding the impact and persistence of

such events in the development and maintenance of the drug-addicted state in both animal models and human addicts, as well as possible effects of different classes of abused substances on chromatin endpoints.

Although epigenetic mechanisms represent attractive candidates to explain long-lasting, and potentially even permanent, alterations in neuronal function following chronic drug exposure, it is still unclear how long after drug exposure these changes in chromatin structure persist. As mentioned previously, many of the genes known to display altered expression levels after repeated drug administration do not remain elevated or repressed during periods of extended withdrawal. Thus, it is important to shift the focus of current research to more thoroughly examine alterations in the transcriptional inducibility of gene targets following drug/cue re-exposure, as well as the epigenetic phenomena underlying such events (LaPlant et al. 2011). Doing so may aid in the identification of novel gene targets, which could prove useful for the development of future drug therapies. Furthermore, it will be very important to examine in far greater detail the influence of drug exposure early in life, for example, in utero and during adolescence, on the epigenetic state of individual genes and on chromatin-modifying enzymes that control global levels of histone and DNA modifications. As noted earlier, such epigenetic modifications are likely to influence an individual's lifetime risk for addiction. Whether a small number of such epigenetic modifications, occurring in germ cells, can be passed on to subsequent generations to influence drug vulnerability remains highly conjectural and requires direct investigation.

High-throughput genomic analyses of chromatin regulation (e.g., ChIP-chip, ChIP-Seq), as well as targeted approaches such as those described throughout this review, will likewise address questions concerning the importance of many other types of chromatin modifications (e.g., sumoylation, nucleosome remodeling and ubiquitination, to name a few) to drug addiction and will aid in future investigations examining the similarities and/or differences between chromatin-mediated gene regulation in response to several drugs of abuse throughout numerous brain regions implicated in addiction. Such studies will allow for a fuller understanding of the ways in which drug-induced gene expression profiles throughout numerous limbic forebrain regions affect neural connectivity and electrophysiological communication between associated structures.

Lastly, it is important to consider that many of the brain regions affected by drug of abuse are not homogeneous but rather are highly heterogeneous structures composed of many distinct cell types with different afferent and efferent connections and electrophysiological properties. For example, although the NAc is composed ~95% of medium spiny, GABAergic projection neurons receiving common inputs from several afferent pathways (e.g., glutamatergic, dopaminergic, etc.), these neurons express distinct dopamine receptor subtypes—dopamine D1 vs. D2 receptors——that promote opposing downstream signaling cascades, leading to very different transcriptional outcomes and, oftentimes, quite distinct behavioral responses to drugs of abuse (Self et al. 1996; Surmeier et al. 2007; Heiman et al. 2008; Bateup et al. 2010; Chen et al. 2010; Lobo et al. 2010). Analogous studies are needed for the several types of interneurons present in the NAc as well as glial cells. Therefore, it

will be necessary to further examine drug-induced chromatin modifications in a cell type-specific manner to more fully understand the contribution of differential modes of transcriptional plasticity in distinct cell types that may be involved in the development of addictive behaviors.

In conclusion, although our understanding of the molecular mechanisms underlying drug addiction remains incomplete, the identification of chromatin remodeling as an important mediator of drug-induced transcriptional, synaptic, and behavioral plasticity represents an exciting new area of research with potential therapeutic benefits. The ability to reverse the epigenetic landscape controlling the addicted state offers an approach that may aid in the development of more effective treatments for addiction, not only through direct targeting of aberrant chromatin regulation but also through the future identification of target genes involved in addiction pathogenesis.

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